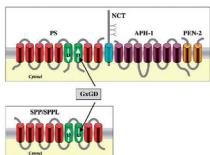Nigel M. Hooper
Uwe Lendeckel

Editors

Intramembrane-Cleaving Proteases (I-CLiPs)

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INTRAMEMBRANE-CLEAVING PROTEASES (I-CLiPs)

PROTEASES IN BIOLOGY AND DISEASE

SERIES EDITORS:

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INTRAMEMBRANE-CLEAVING PROTEASES (I-CLiPs)

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PREFACE

In recent years a growing number of proteases have been identified that catalyse peptide bond hydrolysis in the plane of the cellular membrane. These so-called 'intramembrane-cleaving proteases' (I-CLiPs) are involved in a diverse range of cellular processes, including cell regulation, signalling and protein processing. Some I-CLiPs play critical roles in diseases such as Alzheimer's and viral infection. The aim of this book is to provide an update on this emerging group of unusual but important proteases for both the specialist and those with a broader interest in proteases.

The book begins with a chapter by Rob Rawson and Wei-ping Li on the first I-CLiP to have been recognised as such, the Site-2 protease. This protease, which has the prototypical zinc binding His-Glu-Xaa-Xaa-His motif, plays a crucial role in mammalian lipid metabolism and the unfolded protein response, and therefore is key to both normal and disease processes. Although it is now 10 years since Site-2 protease was discovered, several questions about this protease remain unanswered and these are highlighted.

In Chapter 2, Todd Golde and colleagues introduce the signal peptide peptidases (SPPs), focusing on SPP and SPPL3. These multipass membrane proteins with critical aspartic acid residues in their active site, function as proteases without the need for additional cofactors. Only a few endogenous substrates for SPP have been identified and its biological role is largely unknown. The possibility that the SPPs may be novel antiviral drug targets in humans and represent a novel drug target for major human pathogens, such as malaria, is discussed. This family of GXGD-type intramembrane aspartate proteases, is further expounded upon in Chapter 3 by Harald Steiner and Christian Haass. They focus on the role that SPP plays to clear the ER membrane of signal peptides of secretory proteins, and the role that SPPL2a and b may have in cleaving tumour necrosis factor-α to release an intracellular domain that triggers interleukin-12 signaling.

The intramembrane serine proteases, the rhomboids, are the attention of Chapter 4 by Sinisa Urban. These proteases play key roles in a range of cell communication events, including tyrosine kinase signalling during animal development and quorum sensing during bacterial growth. The first high resolution crystal structure of a rhomboid protease has recently been reported providing new insights into the

structure and mechanism of action of these proteases. In Chapter 5, Elke Pratje continues with a description of the rhomboid family members, Rbd2 and Pcp1, in yeast. Rbd2 is associated with the Golgi, but its function and substrates are unknown, while Pcp1 is located in the inner mitochondrial membrane where it catalyses the second step in the proteolytic processing of cytochrome c peroxidase. PcP1 also affects the morphology of mitochondria by acting on the dynamin-related GTPase, MgM1.

The final two chapters discuss the role of the presenilin/γ-secretase complex in the proteolytic processing of the Alzheimer's amyloid precursor protein and the developmental protein Notch, respectively. In Chapter 6 Michael Wolfe describes the role of the amyloid-β peptide in Alzheimer's disease and the key part played by the γ-secretase complex in this process. The γ-secretase complex consists of four different integral membrane proteins, the presenilins, nicastrin, Aph-1 and Pen-2. Two critical aspartic acid residues in the presenilins constitute the active site of this I-CLiP. The role of the subunits in the maturation of the complex and in the recognition of substrates is discussed, along with the potential for inhibitors and allosteric modulators of γ-secretase activity as potential Alzheimer's disease therapeutics. In Chapter 7, Raphael Kopan and colleagues describe how the convergence of previously independent fields of research led to deciphering the proteolytic mechanism for Notch activation and the role of γ-secretase in its regulated intramembrane proteolysis.

The study of I-CLiPs has emerged as an exciting research area in cell biology, and we trust that this volume in the *Proteases in Biology and Disease* series will prove to be a timely and valuable source of information on these proteases. Finally, we would like to thank all the authors for their scholarly contributions.

Nigel M. Hooper and Uwe Lendeckel

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CHAPTER 1

THE SITE-2 PROTEASE AT TEN

ROBERT B. RAWSON[1] AND WEI-PING LI[2]

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Abstract: The site-2 protease (S2P) is a highly hydrophobic integral membrane protean required for cleavage of various mambrane-bound transcription factors within a membrane-spanning helix. S2P was the first intramembrane-cleaving protease to be recognized but more has been learned about other such proteins. Fundamental questions about the role and function of S2P remain unanswered. S2P plays a crucial role in mammalian lipid metabolism and the unfolded protein response. Thus, finding the answers has implications for our understanding of human health and disease. Recent advances with rhomboid proteins and gamma secretase indicate that the technical challenges to getting the answers can be overcome

Keywords: ATF-6, S2P, SREBP, Regulated intramembrane proteolysis (Rip)

1.   INTRODUCTION

A decade after its discovery, the Site-2 protease (S2P) continues to resist efforts to shed light on some of the most basic questions concerning this enigmatic protein. Despite being the first intramembrane-cleaving protease (I-CLiP; (Wolfe *et al.*, 1999)) to be recognized as such, more has been learned about other, more recently-discovered I-CLiPs.

The evidence that S2P is indeed a protease, for example, rests solely on extensive genetic analysis (Rawson *et al.*, 1997; Zelenski *et al.*, 1999) and on its primary amino acid sequence that has an histidine-glutamate-(any amino acid)$_2$-histidine (His-Glu-*X*-*X*-His) motif. These residues are characteristic of the active site of many metalloproteases (Rawlings and Barrett, 1995). Direct evidence of proteolytic activity, such as cleavage of a substrate *in vitro* by purified S2P, are still lacking. Notwithstanding these caveats, it seems certain that S2P is indeed a protease.

With the available data and in the absence of any evidence to the contrary, we proceed on this widely-held assumption. Indeed, S2P is included in the MEROPS database of proteolytic enzymes ((Rawlings *et al.*, 2006); clan MM, family M50A,

peptidase M50.001) and proteins highly similar in sequence to S2P are found in nearly every genome that has been sequenced. The widespread occurrence of S2P family members suggests that an S2P protein was present in the last common ancestor of current life forms (Kinch *et al.*, 2006). Here we consider what *has* been learned about S2P, and what questions remain unanswered.

2.   BACKGROUND

S2P was discovered as part of the effort to understand the global regulation of lipid metabolism in mammalian cells (Brown and Goldstein, 1999). One of the major systems regulating lipid metabolism is the sterol regulatory element binding protein (SREBP) pathway. The SREBPs are transcriptional activators of genes needed for lipid synthesis and uptake (*e.g.* fatty acid synthase (FAS) and the low density lipoprotein (LDL) receptor gene, respectively). In vertebrates, there are two distinct genes that encode three different isoforms, SREBP-1a, -1c, and -2 (Hua *et al.*, 1993; Yokoyama *et al.*, 1993). When cellular need for lipid rises, SREBPs are activated.

2.1.   Membrane-bound Transcription Factors

An unusual feature of the SREBPs, given that they are transcription factors, is that they are made as precursors that are integral membrane proteins of the endoplasmic reticulum (ER), owing to the presence of two membrane-spanning helices. The precursor adopts a hairpin configuration such that both the amino- and carboxy-terminal domains are cytoplasmic (Hua *et al.*, 1995). Transcriptionally active SREBP is the amino-terminal fragment of the precursor. This fragment, lacking the membrane anchors, is free to enter the nucleus and bind to the sterol regulatory elements (SREs) in the promoters of target genes, resulting in their increased transcription.

The observed production of an active amino-terminal fragment from a membrane-bound precursor indicated that some protease (or proteases) cleaved the precursor to release active SREBP. Further, Wang *et al.* demonstrated that cleavage of the precursor was regulated by sterols (Wang *et al.*, 1994). This revealed part of the mechanism by which sterols regulate their own synthesis in mammalian cells: when sterols are in short supply, SREBPs are cleaved and the transcription of the genes of sterol synthesis and uptake is increased; when sufficient sterols are present, no cleavage occurs, and there is no increase in transcription or synthesis.

Initially, some hypothesized that this was the whole story. Gasic proposed that SREBP, by virtue of its two membrane-spanning helices, sensed the sterol content of the ER membrane directly, perhaps undergoing a conformational change that rendered it susceptible to proteolysis. This then would release the transcriptionally active amino terminus (Gasic, 1994). Further work, however, revealed a much more complex and intriguing mechanism of SREBP activation.

2.2. Two-step Cleavage

For example, Hua *et al.* found that amino acid sequences on each side of the first transmembrane domain of SREBP are required in order for regulated cleavage to occur (Hua *et al.*, 1996). On the luminal side, the arginine residue at amino acid 519, located in the solvent-accessible loop separating the two membrane-spanning helices, was required for cleavage. On the cytoplasmic side of the membrane, the motif Asp-Arg-Ser-Arg in the juxtamembrane region was also required (Hua *et al.*, 1996). When mutated to Ala-Ser, normal cleavage of SREBP 2 cannot occur.

Proteases which cleave extracellular domains of proteins in the juxtamembrane region had been identified previously, such as matrix metalloproteinase-3 (MMP3) that cleaves heparin-binding EGF-like growth factor (Suzuki *et al.*, 1997). None of the substrates identified, however, also required sequences on the opposite side of the membrane in order for cleavage to take place. Thus, either the enzyme that cleaved SREBP was highly unusual (for example, spanning the membrane with two active sites on different sides, or possessing a single site that traversed the membrane), or two separate proteases were needed to release the amino terminal domain of SREBP. These unanticipated findings made identification of the cleavage machinery all the more important.

2.3. Isolating S2P

In order to identify S2P, workers in the laboratory of Mike Brown and Joe Goldstein continued with a complementation approach that had been initiated in the laboratory of T.Y. Chang at Dartmouth (Hasan *et al.*, 1994). Chang and co-workers isolated mutant Chinese hamster ovary cell lines that failed to upregulate the transcription of the genes of cholesterol synthesis and uptake in the face of increased demand. These mutant cells therefore required medium supplemented with cholesterol and unsaturated fatty acid (oleate) in order to grow (Hasan *et al.*, 1994). One of these lines, designated M19, was selected for complementation cloning.

In order to circumvent potential pitfalls of complementation using cDNAs, high molecular weight genomic DNA from human cells was used to transfect the M19 cells (Fig. 1). Those cells that regained the ability to grow in unsupplemented medium had received a functional copy of the gene they lacked from the transfected human DNA. Of course, those cells had received other fragments of human DNA in addition to the one that restored the mutant function. Some of these irrelevant human sequences also were stably integrated into the genomes of the rescued cells.

Genomic DNA from the complemented mutant cells was used once more to transfect mutant M19 cells (Fig. 1). This served to partially 'purify' the human DNA sequences encoding the rescuing activity. All complemented cells must have in common the rescuing DNA. On the other hand, the extraneous human DNA sequences that were fortuitously integrated into the genomes of the rescued cells would vary from line to line. Each successive round of transfection reduced the likelihood that any given stretch of irrelevant, non-rescuing, human DNA would be
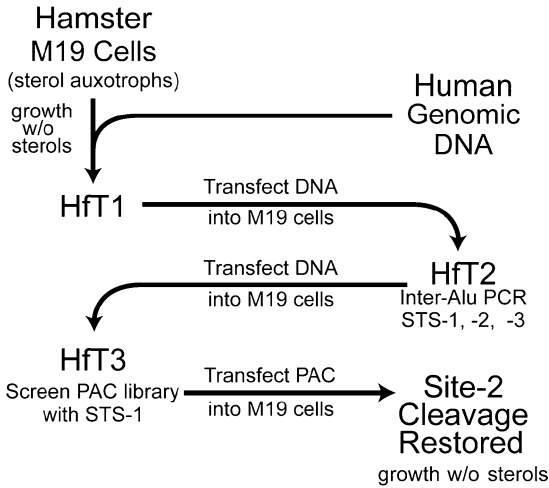
Figure 1. Steps in cloning S2P. Mutant **M19** Chinese hamster ovary cells fail to upregulate the genes of cholesterol and unsaturated fatty acid synthesis and require free cholesterol added to the medium in ethanolic solution in order to grow (Hasan and Chang, 1994). M19 cells were transfected with high molecular weight genomic DNA prepared from human cells. The transfected cells were then cultured in medium without added free cholesterol. Under these conditions, the mutant cells die while cells with wild-type function survive. One surviving clone, designated **HfT1**, was selected for further study. Genomic DNA from rescued HfT1 cells was transfected onto M19 cells and the selection repeated. The resulting clone was designated **HfT2** (Hasan *et al.*, 1994). The process was repeated using genomic DNA prepared from HfT2 cells to yield the **HfT3** cells. Inter-Alu PCR was used to amplify human sequences from HfT2 cells and unique human sequences were identified, designated **STS-1**, **-2**, and **-3** (Rawson *et al.*, 1997). Primers specific for STS-1, -2, and -3 were used to screen the rescued mutant cells. STS-1 was common to all rescued cells. STS-1 was used to screen a BAC library and the resulting human genomic DNA clone was transfected into M19 cells. The growth of the PAC-transfected mutant cells in the absence of added cholesterol confirmed that all sequences necessary for rescue were included within the PAC clone. Sequencing and BLAST searches lead to the identification of a human cDNA that also rescued mutant cells, S2P (Rawson *et al.*, 1997)

stably integrated in the rescued mutants. This made possible the identification of the rescuing gene.

The Alu family of ~300 base-pair dispersed middle repetitive sequences is abundant in the human genome (Schmid and Jelinek, 1982) but is not found in rodents. Thus Alu sequences serve as markers of human DNA. We employed the technique of inter-Alu PCR (Nelson *et al.*, 1989) to isolate probes for unique human DNA sequences present in the rescued mutant cells (Rawson *et al.*, 1997). One of these probes, STS-1, identified sequences unique to the human DNA found in each generation of the rescued mutant cells (Rawson *et al.*, 1997). This probe was used to screen a library of human genomic DNA cloned into a bacterial artificial chromosome (BAC) vector. The ~ 100 kb BAC clone thus isolated also rescued when transfected onto mutant M19 cells (Fig. 1). This confirmed that this clone encoded all sequences required for rescue of the mutant cells.

Sequence analysis of this clone lead to the identification of a human X chromosome gene (and the consequent isolation of its cDNA) that encoded a previously unknown protein. While its function was also unknown, the predicted amino acid sequence contained an His-Glu-*X*-*X*-His motif, which suggested that the unknown protein was a metalloproteinase.

2.4.   A Protease?

As mentioned above, site-directed mutagenesis and transfection studies of the cDNA (including rescue assays - the restoration of SREBP cleavage to cells lacking S2P) demonstrated that both the histidines and the glutamate were necessary in order to restore cleavage of SREBP to mutant cells (Rawson *et al.*, 1997). This evidence strongly supported the conclusion that this protein was a protease that cleaved SREBPs and thus it was designated S2P (site-2 protease). Unusually for a protease, S2P is extremely hydrophobic; 43% of its residues are non-polar amino acids (Ala, Ile, Leu, Phe, Trp and Val). In fact, S2P and its homologues are the most hydrophobic proteases yet described. Its primary structure includes numerous sequences predicted to be membrane-embedded helices (Zelenski *et al.*, 1999). Biochemical studies confirmed that S2P is an integral membrane protein (Rawson *et al.*, 1997). Despite substantial effort, the difficulties encountered in reconstituting the activity of a membrane-imbedded enzyme against its membrane-imbedded substrate have not been overcome for S2P. No assay employing purified S2P or purified substrate has been reported. Thus, direct demonstration of proteolytic activity is lacking for S2P and it is unknown whether it requires any cofactors in order to function. This situation may not remain for long.

Successful efforts for the development of *in vitro* assays have been reported for Rhomboid family intramembrane proteases (Lemberg *et al.*, 2005; Urban and Wolfe, 2005) and gamma secretase (Fraering *et al.*, 2004; Hoke *et al.*, 2005). Thus it is possible to reconstitute the activity of at least some intramembrane proteases *in vitro,* and future efforts with S2P may prove availing.

3.   STRUCTURE AND FUNCTION

3.1.   Membrane Topology of S2P

In the absence of in vitro assays, much of what is known about S2P comes from studies in mammalian cells. Zelenski *et al.* determined the topology of S2P with respect to the membrane by a combination of epitope tagging and protease protection assays as well as access of portions of S2P to the glycosylation machinery in the lumen of the ER (Zelenski *et al.*, 1999). The proposed topological model supported by experimental evidence (Fig. 2) differs from that derived from algorithmic analysis (Lewis and Thomas, 1999).

S2P contains three regions of significant hydrophilicity: (1) residues 21–70 (numbered according to the human sequence); (2) residues 108–141, comprised
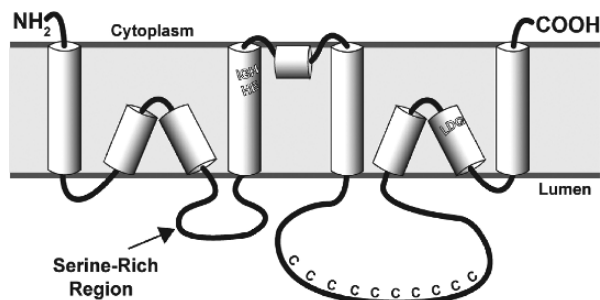
Figure 2. Membrane topology of S2P. This cartoon represents a model of the proposed membrane topology of S2P. The cytoplasmically-disposed amino- and carboxy-termini are indicated. The solvent-accessible loops (*e.g.* Ser-rich region, Cys-rich region) are in the lumen. The hydrophobic sequences are most likely embedded in the bilayer (grey box). The proposed location of the active site residues (HEIGH, LDG) are shown. The disposition of hydrophobic sequences within the bilayer is inferred from the location of the solvent-accesible sequences. Cylinders represent probable alpha helices. This model is based on data from (Kinch *et al.*, 2006; Zelenski *et al.*, 1999)

of multiple Ser residues of undetermined function; there are 26 Ser in this region of human S2P but varying numbers in other species, and (3) residues 258–446, including 12 Cys residues conserved in all S2P orthologues. These hydrophilic regions reside in the luminal space (Fig. 2). The amino- and carboxy-termini face the cytoplasm. Several of the predicted membrane-spanning helices do not cross the bilayer; the hydrophilic regions on either side of them can be glycosylated and thus must be disposed in the lumen (Zelenski *et al.*, 1999). If sequences on either side are luminal, then the hydrophobic sequence cannot span the bilayer. Instead, these sequences must 'dip' into the membrane with the peptide chain reemerging on the same side of the membrane as it entered.

Other intramembrane proteases are broadly similar. For example, the recently-solved crystal structure of the Rhomboid protein GlpG from *E. coli* shows that it also has helices that lie within the plane of the bilayer without crossing it as well as having additional membrane-associated sequences (Wang *et al.*, 2006; Wu *et al.*, 2006). Wang *et al.* and Wu *et al.* suggest that various of these may serve a gating function to regulate substrate access to the active site. It will be interesting to see if similar structural features are present in S2P, bearing in mind that the two protein families are completely unrelated by sequence.

The available data afford one more inference about the topology of S2P: if Asp_{467} is the third residue coordinating the zinc atom of the active site, then the carboxy-terminal region of S2P must bend back to the amino-terminal portion. This would enable membrane embedded regions of the protein that are distant from one another in the linear sequence of the protein to come together to form the active site.

3.2.   Other Substrates

ATF-6α and β activate the transcription of genes of the unfolded protein response (Yoshida *et al.*, 1998). These membrane-bound transcription factors also need to be cleaved in order to release a transcriptionally active fragment from the precursor (Haze *et al.*, 1999; Yoshida *et al.*, 2000). Just as for the SREBPs, this processing requires the sequential action of S1P and S2P (Ye *et al.*, 2000b). Recently, Murakami *et al.* reported evidence that a protein related to ATF-6, which they refer to as old astrocyte specifically induced substance (OASIS; also known as cAMP responsive element binding protein 3-like 1), also requires S1P- and S2P- mediated proteolysis for its transcriptional activity (Murakami *et al.*, 2006).

They identified a potentially helix-destabilizing Pro residue (see below) at position 391 within the membrane-spanning helix, as well as a site that conforms to the S1P Arg-*X*-*X*-Leu consensus (Arg$_{421}$-Ser-Leu-Leu). These motifs are conserved in OASIS homologues from other organisms, including zebrafish (RBR, unpublished observations). It seems probable that OASIS is indeed a substrate of S1P and S2P. However, the compelling evidence that mutant cells that lack S1P and S2P fail to process OASIS has not been produced and explanations for these data other than those offered by Murakami *et al.* are possible.

3.3.   Mechanism

3.3.1.   *Substrate selectivity*

All known substrates of S2P are type II membrane-spanning domains (amino-terminus cytoplasmic, carboxy-terminus extra-cytoplasmic (e.g. the lumen of the ER)). This includes the SREBPs (once cleavage has taken place at site-1) and activating transcription factors (ATF) -6α and –β (see below). Restricted substrate orientation seems to be a general feature of intramembrane cleaving proteases. The presenilins, which are aspartyl proteases, cleave type I membrane-spanning helices while the other class of aspartyl I-CLiPs, the signal peptide peptidase family, cleaves type II (Weihofen *et al.*, 2002). Rhomboid proteases seem to be restricted to type I substrates.

An exception to this general specificity for substrate topology within the S2P family is SpoIV FB. This protease from *Bacillus subtilis* cleaves a type I substrate. Its predicted topology is opposite that of mammalian S2P and its active site is therefore disposed in the opposite sense relative to the cytoplasm (Rudner *et al.*, 1999). This may explain the alternative orientation of its substrates. A similar situation applies to the membrane topology of signal peptide peptidase (type II substrates) and presenilins (type I substrates) whose active sites are disposed in the opposite sense relative to the membrane (Friedmann *et al.*, 2004).

3.3.2.   *Active site*

In the absence of in vitro data, clues to the mechanism of intramembrane proteolysis by S2P are indirect. In well-studied metalloproteases such as thermolysin,

the two histidines help to coordinate the zinc atom while the glutamate residue activates a water molecule that makes a nucleophilic attack on a scissile bond. Many metalloproteases also have an additional coordinating residue (aspartate, glutamate, histidines, or tyrosine) at varying distance from the His-Glu-X-X-His motif. In S2P, an aspartate residue within the sequence Leu_{466}-Asp-Gly, which lies 290 amino acids after the His_{171}-Glu-Ile-Gly-His motif, appears to serve this role (Zelenski *et al.*, 1999). Mutation of either His or the Glu of the His_{171}-Glu-Ile-Gly-His sequence or of the aspartate of the Leu_{466}-Asp-Gly sequence abolishes the ability of an S2P cDNA to restore SREBP cleavage in the mutant M19 cells (Zelenski *et al.*, 1999).

3.3.3.   Unwinding the substrate

The site of cleavage of SREBP-2 by S2P is between Leu_{484}-Cys. These residues lie within the first membrane-spanning helix, close to the cytoplasmic face of the bilayer (Duncan *et al.*, 1998). Interestingly, although the Leu-Cys motif is found in all SREBP homologues, either one or both of these residues may be substituted by other amino acids without detectable effect on cleavage by S2P (Ye *et al.*, 2000a). Ye *et al.* showed that the Asp-Pro motif in the middle of the substrate membrane-spanning helix was crucial for cleavage by S2P. When those residues are mutated to Phe-Leu, cleavge by S2P is abolished (Ye *et al.*, 2000a). They proposed a model whereby this motif functions as an amino-terminal cap for part of the membrane spanning helix, allowing the remainder of the helix to partially unwind and expose the scissile bond to S2P. This notion is further supported by the presence of a similar motif (Asn_{391}-Tyr-Gly-Pro) in the membrane-spanning helix of the ATF-6α and -β proteins. When either the Asn or Pro residues are altered, cleavage is largely unaffected. Substitution of both residues, by contrast, abolishes ATF-6 cleavage by S2P (Ye *et al.*, 2000b). Subsequently, similar helix-destabilizing motifs were identified as important features of the substrate for other I-CLiPs such as rhomboid and the signal peptide peptidase (Lemberg, 2003; Urban, 2003). Membrane-associated polypeptide chains are thought to favor alpha helical structures and peptide bonds within an alpha helix are refractory to hydrolysis (Paetzel *et al.*, 1998). Thus, partial unwinding of substrate helices to permit hydrolysis may be a general feature of intramembrane proteolysis.

3.3.4.   PDZ domain

Mammalian S2P contains sequences having similarity to so-called PDZ domains. Extensive sequence and structural analysis suggests a functional role for this feature (Kinch *et al.*, 2006), perhaps in the recognition/binding of the newly-generated carboxy-terminus resulting from cleavage of the substrate by S1P. As with other notions of S2P function, direct biochemical demonstration of this hypothesis is lacking. Kinch *et al.* also identified two highly conserved motifs that may serve a role in substrate binding (Kinch *et al.*, 2006).

A significant challenge to understanding the mechanism of intramembrane proteolysis is the suggestion that hydrolysis occurs within the plane of the bilayer. There

are substantial thermodynamic costs to extracting a membrane-spanning helix from the bilayer and into the aqueous environment. These costs are greatly reduced if only a portion of a helix need be extracted. It is worth keeping in mind that S2P, like the other I-CLiPs, is a rather large protease (519 amino acids in human). S2P may be capable of forming a local environment within the bilayer where both helix unwinding and hydrolysis are thermodynamically favorable.

The recently reported structures of rhomboid family proteases suggests such a possibility (Ben-Shem *et al.*, 2007; Del Rio *et al.*, 2007; Lemieux *et al.*, 2007; Wang *et al.*, 2006; Wu *et al.*, 2006). Other I-CLiPs may employ a similar strategy for cleaving membrane spanning helices. Again, resolving this question for S2P will require structural and enzymological data that are currently unavailable. To date, there is no evidence suggesting that S2P requires any posttranslational modification for activation. This contrasts with other soluble or membrane-associated proteases (e.g. Caspases, kexin) which are synthesized as proenzymes or preproenzymes and require removal of an inhibitory segment prior to activation.

The unusual features of S2P's substrates, which are membrane- spanning helices having short carboxy termini in an extra-cytosolic compartment, may limit its activity sufficiently to prevent it from cleaving inappropriate substrates. Protecting the cell from errant S2P activity then becomes the responsibility of a protease that *is* synthesized as a proenzyme, S1P (Espenshade *et al.*, 1999).

3.3.5. *Localization*

S2P is a Golgi enzyme (Fig. 3). This localization was suggested by studies that demonstrated that S1P resides in the Golgi apparatus (DeBose-Boyd *et al.*, 1999) and that the product of SREBP cleavage by S1P (the intermediate form) does not accumulate appreciably in normal cells (Sakai *et al.*, 1996). This suggests that S2P cleaves SREBP immediately subsequent to S1P cleavage. The simplest hypothesis, then, is that S2P also resides in the Golgi. Studies of over-expressed, epitope-tagged S2P supported this notion (Shen and Prywes, 2004).

In order to avoid potential artifacts arising from overexpression or alteration of the protein sequence, we sought to localize endogenous S2P in cultured cells. Using previously described techniques (Daniels *et al.*, 1985), we prepared monoclonal antibodies in mice immunized with antigen corresponding to the Cys-rich luminal loop of human S2P (residues 264-420; Fig. 3A). When used in immunoblotting analysis, these antibodies recognize a band present in wild-type cells but not in mutant M19 cells that harbor a deletion of the S2P gene (Fig. 3B). This demonstrates specificity for S2P. Note that the mutant cells lacking SCAP and S1P were derived from wild-type CHO cells stably over-expressing multiples copies of a human S2P cDNA transgene (Rawson *et al.*, 1998; Rawson *et al.*, 1999). This accounts for the intensely reacting S2P bands in those extracts (Fig. 3B).

The anti-S2P antibodies proved suitable for immunofluorescence studies as well. Fig. 3C shows the immunolocalization of S2P in human SV589 cells (Fig. 3C, upper panels). S2P colocalizes with wheat germ agglutinin (WGA), an established marker for the Golgi apparatus. The mouse monoclonal anti-S2P antibody shows
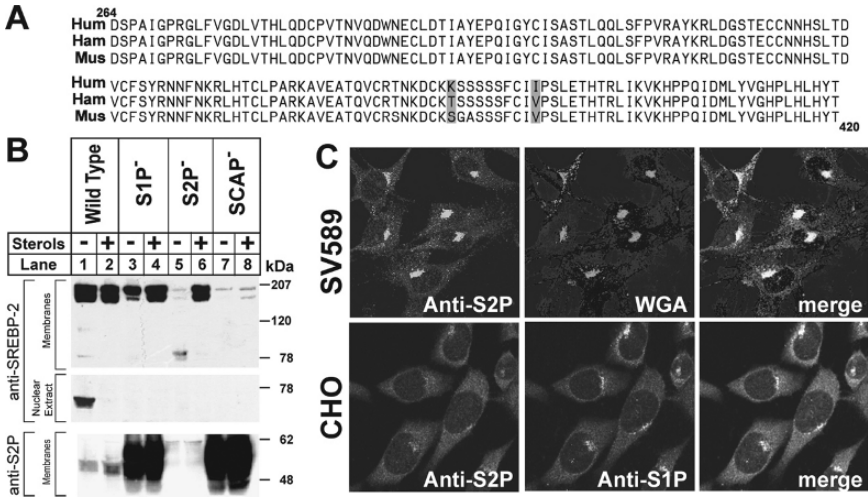
Figure 3. Golgi localization of endogenous S2P. Immunolocalization of S2P in mammalian cells was achieved by raising monoclonal antibodies from mice immunized with antigen corresponding to the protein fragment shown in (**A**). (**B**) Specificity of the antibody was confirmed by western blotting of membrane fractions and nuclear extracts from wild-type and various mutant cell lines. The *SCAP⁻* and *S1P⁻* mutants, which cannot cleave SREBPs, were derived from wild-type Chinese hamster ovary cells over-expressing human S2P. These mutants therefore show much greater accumulation of S2P in their membranes as compared to wild-type cells. The parental, stably-transfected cells show indistinguishable levels of S2P (not shown). (**C**) In human SV589 cells, anti-S2P colocalizes with wheat germ agglutinin (see merge). In Chinese hamster ovary cells, anti-S2P colocalizes with S1P (see merge). In mutant M19 cells that lack S2P, only background levels of fluorescence (without the characteristic Golgi pattern) are observed (not shown). Anti-S1P polyclonal antibody is described in (Espenshade *et al.*, 1999)

apparently weaker affinity for rodent (hamster, mouse) S2P than for human, both in western blot experiments as well as in immunofluorescence. For example, note the higher level of background fluorescence in the CHO cells stained with anti-S2P (Fig. 3C, lower panels). This differential affinity was unexpected since mouse, human, and hamster S2P are highly similar in sequence in the region used as an antigen (Fig. 3A). Notwithstanding, immunolocalization in Chinese hamster ovary cells demonstrated that S1P and S2P colocalize with one another (Fig. 3C, lower panels). As S2P colocalizes with wheat germ agglutinin, these results confirm the inference from earlier studies; S2P is a Golgi enzyme.

4.   REGULATED INTRAMEMBRANE PROTEOLYSIS (RIP)

The identification of two-step cleavage of SREBPs and isolation of S2P came at a time when investigations into Alzheimer's disease suggested that the amyloid precursor protein (APP) was also subject to sequential cleavage, including one

within its membrane-spanning helix. These data, and others, lead to the recognition of a previously-unappreciated mechanism of cellular signaling – Rip (Brown *et al.*, 2000). Rip involves cleavage of a substrate within a membrane-spanning helix to release a molecule that functions in signaling. Since its initial description for the SREBP pathway and APP processing, Rip has been identified in a number of other systems as described elsewhere in this volume, ranging from the unfolded protein response (Ye *et al.*, 2000b) to growth and development (De Strooper *et al.*, 1999; Lee *et al.*, 2001; Selkoe and Kopan, 2003; Urban *et al.*, 2001).

5.   OUTSTANDING QUESTIONS

As initially noted, there remains much to learn about S2P. For example, does S2P require any small molecule cofactors such as ATP? Is there any control of its activity by post-translational modification. Glycosylation of its Cys-rich loop is dispensable but nothing is known about possible phosphorylation of S2P, for example.

Are additional proteins needed for its function? There is precedent from other S2P family members. In the case of SpoIVFB from *Bacillus subtilis* that cleaves pro-σ^k, two additional proteins, BofA and SpoIVFA, provide negative regulation (Cutting *et al.*, 1991). Regulation of SpoIVFA activity is in turn accomplished by two different soluble serine proteases, SpoIVB and CtpB. Either enzyme can cleave SpoIVA, releasing its inhibition of SpoIVFB (Campo and Rudner, 2006). Might an analogous system exist for S2P? If so, it would likely involve currently unknown substrates. This brings up the next question: are there unrecognized substrates for S2P?

The answers to these questions will in part depend on the development of an *in vitro* assay for S2P activity. The cell-based assays currently available show that S2P is necessary for proteolysis of SREBPs and ATF-6s; they cannot address sufficiency.

S2P is essential in mammalian cells cultured in medium prepared with lipoprotein- or lipid-deficient serum (Evans and Metherall, 1993; Hasan and Chang, 1994; Rawson *et al.*, 1997). On the other hand, mice completely lacking Site-1 protease die during embryogenesis (Yang *et al.*, 2001), perhaps as a result of deficient processing of SREBPs or ATF-6s. Is S2P similarly essential in the whole animal?

Other much-desired information includes structural data. Koide *et al.* used alkylation of Cys residues to probe the structure of the active site of RseP, an S2P orthologue form *E. coli* (Koide *et al.*, 2006). Their data indicate that the active site lies within the plane of the bilayer, in accordance with predictions, and is at least partially accessible to the aqueous phase. As with topology data, these results are tantalizing, leaving us wanting more. The recent spate of rhomboid structures demonstrates that such data can be gathered for intramembrane cleaving proteases (Ben-Shem *et al.*, 2007; Del Rio *et al.*, 2007; Lemieux *et al.*, 2007; Wang *et al.*, 2006; Wu *et al.*, 2006). Many of the speculations about S2P here presented can be suitably addressed by detailed structural information.

6.   CONCLUSIONS

Isolation of S2P, the first I-CLiP to be identified, lead to the recognition that intramembrane proteolysis is a widespread mechanism of cell signaling. Beside this role, S2P is an interesting molecule in its own right. Ten years after its discovery, however, many basic issues of S2P biology and enzymology remain unsettled. Recent progress with other intramembrane proteases encourages the speculation that the data required to settle these issues will be forthcoming well before another decade has passed.

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CHAPTER 2

SIGNAL PEPTIDE PEPTIDASES

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Abstract: Signal peptide peptidases (SPPs) are the most recently identified members of a protease family of integral membrane proteins that includes the intensively studied presenilin 1 (PS1) and presenilin (PS2) proteins. There are 5 human genes encoding SPPs which can be divided into two branches based on homology and initial functional studies. One branch, which is the focus of this chapter, consists of the SPP and SPPL3 proteins. The second branch will be the focus of a subsequent chapter, and consists of the three SPPL2 proteins (SPPL2a, b, and c). The SPP proteins are conserved through evolution with family members found in fungi, archaea and plants. Presenilins (PSs) and SPPs cleave substrate polypeptides within a transmembrane region, but differ in that PSs cleave type 1 membrane proteins whereas SPPs cleave type 2 membrane proteins. SPPs and PSs have low overall sequence homology, yet exhibit considerable structural similarity as well as strict conservation of several small motifs. They are both multipass membrane proteins that contain two conserved active site motifs YD and GxGD in adjacent membrane-spanning domains and a conserved PAL motif of unknown function near their C-termini. They differ in that the active site topology of SPPs is inverted relative to PSs. Moreover, SPP and SPPL3 appear to function as proteases without the need for additional cofactors. In contrast, PSs function as the γ-secretase protease only when complexed with three accessory proteins. Although the biological roles of PSs are reasonably well understood, the biological roles of SPP are largely unknown, and only a few endogenous substrates for SPP have been identified. SPP and possibly SPPL3 appear to cleave a number of endogenous type 2 signal peptides and these genes are essential genes in the development of several model organisms. In addition, in many human parasites, there is only a single SPP gene that is most closely related to the human SPP. Thus, SPPs may be novel antiviral drug targets in humans and represent a novel drug target for major human pathogens such as malaria

Keywords: protease, intramembrane proteolysis, signal peptide peptidase

1.   INTRODUCTION: INTRAMEMBRANE CLEAVING PROTEASES

Intramembrane cleaving proteases (I-CLiPs) are defined as enzymes that catalyze the cleavage of peptide bonds within the plane of the lipid bilayer. Three families of I-CLiP-s have been identified. The active sites of these proteases appear to lie

within the plane of the lipid bilayer, yet they use catalytic mechanisms analogous to those of soluble proteases with active sites that operate in an aqueous environment. The original I-CLiP and prototypic member of the first family is the human site-two protease (S2P) that cleaves and activates the sterol regulatory element binding proteins (SREBPs) (Rawson *et al.* 1997). Based on conservation of residues found to be essential for its activity, S2P appears to use a catalytic mechanism similar to classic metalloproteases (Rudner *et al.* 1999; Ye *et al.* 2000; Kinch *et al.* 2006). The Rhomboids form the second family (Urban *et al.* 2001). The prototypic member of this family is Drosophila Rhomboid-1, which cleaves and liberates several epidermal growth factor (EGF) ligands (Lee *et al.* 2001; Tsruya *et al.* 2002). Based on conservation of residues essential for activity and inhibitor sensitivity, Rhomboids appear to use a catalytic mechanism similar to serine proteases (Urban *et al.* 2001; Gallio *et al.* 2002; McQuibban *et al.* 2003). The recent crystal structure of the *Escherichia coli* Rhomboid GlpGd demonstrates that Rhomboid-mediated catalysis resembles classical serine proteases in two key ways: it uses the amino acid serine to attack the substrate peptide bond, and this serine is activated by interaction with a neighboring histidine (Wang *et al.* 2006b). Moreover, these data conclusively show that the active site lies in the lipid bilayer (Wang *et al.* 2006b). The prototypic members of the third family are i) the presenilins (PSs) involved in cleavage of numerous substrates including the amyloid β protein precursor (APP) and Notch and ii) signal peptide peptidase (SPP) which cleaves signal peptides of type 2 membrane proteins. Mutational studies, sequence alignments, and inhibitor studies suggest that PSs and SPP appear to use a catalytic mechanism similar to classic aspartyl proteases (De Strooper *et al.* 1999; Wolfe *et al.* 1999; Weihofen *et al.* 2002). As other chapters in this book focus on other intramembrane cleaving proteases, this chapter will focus on signal peptide peptidase and its closest homolog, SPPL3.

2.   SIGNAL PEPTIDE PEPTIDASES

Signal peptide peptidases (SPPs) were originally identified *in silico* through a homology search based on the conserved active site motifs of the presenilins (PSs) (Ponting *et al.* 2002), and in the absence of functional data were named "presenilin homologs". Shortly thereafter, one of these "presenilin homologs" was shown to possess proteolytic activity, carrying out intramembrane proteolytic processing of signal peptides of major histocompatability complex class I molecules following cleavage by signal peptidase (Weihofen *et al.* 2002). SPPs are known by various nomenclatures (Table 1) with the signal peptide peptidase nomenclature being the most generally accepted. Accordingly, we will use this terminology in this review. There are five human genes encoding SPPs which, as discussed in detail below, can be divided into two groups based on homology and initial functional studies (see Table 1). One group, which is the focus of this chapter, consists of the SPP and SPPL3 proteins. The second, which will be the focus of a subsequent chapter, consists of the three SPPL2 proteins (SPPL2a,b,c).

   Despite having limited areas of direct sequence identity, human PSs and human SPPs are membrane proteins that exhibit an overall conservation of structural motifs

Table 1. Human SPP nomenclature

Family name	SPP[A]	PSH[B]	IMPAS[C]	PS-Like[D]
Specific Names	SPP	PSH3	IMP1/IMPAS	PSL3
	SPPL3	PSH1	IMP2	PSL4
	SPPL2a	PSH5	IMP3	PSL2
	SPPL2b	PSH4	IMP4	PSL1
	SPPL2c	PSH2	IMP5	

[A](Weihofen *et al.* 2002; Friedmann *et al.* 2004), [B](Ponting *et al.* 2002), [C](Grigorenko *et al.* 2002), [D]NCBI Search.

(Ponting *et al.* 2002). These proteins share identical putative active site motifs, YD and LGLGD (Weihofen *et al.* 2002). In addition, they contain a third conserved motif, PAL, near their C-termini (Wang *et al.* 2006a) (Fig. 1). The YD and LGLGD motifs, which in PSs and SPPs contain the catalytic aspartate residues, are distinctive in that they are present within predicted adjacent and opposing transmembrane regions (Golde and Eckman 2003; Martoglio 2003). However, it is now clear from topology studies that the orientation of these transmembrane regions is inverted in all human SPP family members relative to PSs (Weihofen *et al.* 2002; Friedmann *et al.* 2004; Nyborg *et al.* 2004a). This inversion of the active site appears to have functional consequences as SPPs cleave within the transmembrane domains of type 2 membrane proteins whereas PSs cleave within the transmembrane domains of type 1 membrane proteins. The similarities between PSs and SPPs, particularly in the active site motifs, point to a common catalytic mechanism. As in PSs, mutation of the conserved aspartates in the YD and LGLGD motif appears to abolish proteolytic activity (Weihofen *et al.* 2002; Okamoto *et al.* 2004; Sato *et al.* 2006).

In addition, certain γ-secretase inhibitors that bind and inhibit PS bind and inhibit SPP and certain SPP inhibitors inhibit PS dependent γ-secretase activity (Lemberg and Martoglio 2002; Nyborg *et al.* 2004b; Sato *et al.* 2006). However, given the sequence divergence outside of the conserved active site motifs, one would expect that not all γ-secretase inhibitors would inhibit SPP and vice versa. This appears to be the case; for example, the SPP inhibitor (Z-LL)$_2$ ketone shows marked selectivity for inhibition of SPP over γ-secretase while the γ-secretase inhibitor DAPT does not (Lemberg and Martoglio 2002; Nyborg *et al.* 2004a). Further, the DAPT-related compound LY-411,575 preferentially inhibits γ-secretase but also shows activity against SPP at higher concentration. The mechanistic resemblance of PSs and SPPs is strengthened by the observation that SPPs, like PSs, cleave substrates at multiple sites within the transmembrane domain (Fluhrer *et al.* 2006; Sato *et al.* 2006). Moreover, select compounds are able to alter the relative utilization of cleavage sites rather than merely inhibit overall proteolytic activity. This is a distinctive feature of PS-mediated proteolysis and it further reinforces the mechanistic similarity of PS and SPP-mediated catalysis.

One of the major differences between SPP and PS is that SPP is active in the absence of additional co-factors or subunits (Weihofen *et al.* 2002; Sato *et al.* 2006). It is not yet clear whether other SPP family members (SPPL3 and SPPL2a,b,c)
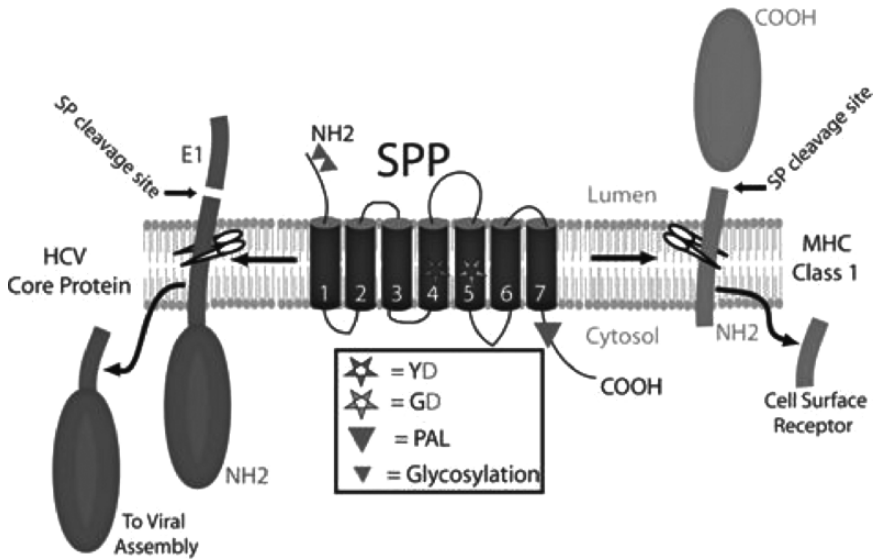
Figure 1. A Schematic diagram of SPP and its role in cleavage of two substrates (HCV core protein and MHC class I) is shown. The box highlights structural motifs found in SPP. The stars refer to the active site aspartates, the large triangle the COOH-terminal PAL motif and the small triangles N-linked glycosylation sites. SP refers to cleavage by signal peptidase

function without co-factors. PSs functions as the catalytic core of γ-secretase only when complexed with three additional proteins (Yu *et al.* 2000; Francis *et al.* 2002; Edbauer *et al.* 2003; Kimberly *et al.* 2003; Marlow *et al.* 2003; Takasugi *et al.* 2003). Two of these proteins Aph-1 and Pen-2 seem to be required for assembly and stabilization of an active γ-secretase complex (LaVoie *et al.* 2003; Fraering *et al.* 2004). The fourth component of the γ-secretase complex, Nicastrin is quite interesting in that it seems to function in substrate recognition, binding the N-terminus of the membrane bound stubs of γ-secretase substrates (Shah *et al.* 2005). Like PS dependent γ-secretase, SPP seems to require a prior cleavage of the substrate to generate a small membrane bound stub (Lemberg *et al.* 2001). If SPP does indeed function without co-factors, then it will be interesting to understand how its activity and substrate specificity are regulated by sequences present entirely within the SPP polypeptide. Of course, it is still possible that an unknown Nicastrin-like co-factor is required for substrate presentation within the membrane but it must be present in all of the *in vivo* systems currently used to assay SPP function.

2.1.   Monomer, Dimer, or Both?

SPP was originally identified as a ∼45 kDa N-linked glycoprotein using an inhibitor labelling approach (Weihofen *et al.* 2002). This size is consistent with a full-length glycosylated monomer. Other reports of solubilized SPP describe it as running as

two bands, one at ∼42 kDa, and one at ∼95 kDa (Grigorenko *et al.* 2002; Nyborg *et al.* 2004b). Under mild lysis conditions, SDS-PAGE, and Western blotting SPP is primarily detected as a ∼95 kDa homodimer (Nyborg *et al.* 2004b). The SDS-stable homodimer is dissociable to a monomer by heating in the presence of SDS and reductant (Nyborg *et al.* 2004b). In addition, an N-terminally FLAG epitope-tagged SPP construct co-purifies with a C-terminally V5 His epitope-tagged SPP construct (Nyborg *et al.* 2004b). These biochemical studies suggest that SPP likely exists as a dimer *in vivo*. However, *in vitro* studies show that detergent solubilized monomeric SPP is capable of cleaving exogenous synthetic peptide substrates (Sato *et al.* 2006). Although the bulk of the evidence would suggest that the majority of SPP and its closest homolog, SPPL3 (Nyborg *et al.* 2006a), are present within the cell as homodimers, it is unclear whether the monomer or dimer represent the active form. Indeed, the *in vitro* data suggest that an SPP monomer can possess proteolytic activity, but this does not mean that the monomer is the active form *in vivo*. Current efforts to obtain more detailed structural information on SPP will hopefully lead to confirmation of its active form.

2.2.   Subcellular Localization

Subcellular fractionation and immunocytochemical localization demonstrate that SPP is predominantly localized to the endoplasmic reticulum (ER) (Urny *et al.* 2003; Friedmann *et al.* 2004; Casso *et al.* 2005; Krawitz *et al.* 2005; Dev *et al.* 2006; Friedmann *et al.* 2006; Urny *et al.* 2006). Similarly, SPPL3 appears to be largely restricted to the ER. This contrasts with SPPL2b which is localized to the endosome and plasma membrane. As known substrates of SPP are also found in the ER, it is hypothesized that SPP functions proteolytically within the ER (Okamoto *et al.* 2004; Dev *et al.* 2006; Hope *et al.* 2006; Targett-Adams *et al.* 2006). Though SPP does contain a putative C-terminal KKXX ER retention signal (Weihofen *et al.* 2002), this signal does not always retain SPP in the ER and is also not required for activity (Nyborg *et al.* 2004a; Nyborg *et al.* 2004b; Casso *et al.* 2005). Further, epitope tags placed after the KKXX ER retention signal do not alter dimer formation or activity (Nyborg *et al.* 2004a; Nyborg *et al.* 2004b; Casso *et al.* 2005). Not all naturally occurring SPP species are confined to the ER (Urny *et al.* 2006). Recently, a splice variant of SPP lacking the ER retention signal (SPPβ) was shown to be located primarily in the plasma membrane (Urny *et al.* 2006). The question of exactly where SPP functions in the cell is still very much an open one. Determining the exact subcellular location or locations of cleavage may take some time to resolve. This effort may well be complicated by the possibility that, like PSs, the majority of SPP is in an inactive state or form. Defining where cleavage occurs in the cell by using methods based on detection of total SPP to infer the location of active SPP therefore may be misleading (Cupers *et al.* 2001).

Although the subcellular localization of SPP and SPPL2b is distinct (Krawitz *et al.* 2005; Friedmann *et al.* 2006), when analyzed by sucrose flotation density gradients, both proteins fractionate into buoyant membrane microdomains which

are often referred to as lipid rafts or detergent resistant membranes (Nyborg *et al.* 2006a). This localization in the buoyant membranes is quite similar to the localization of γ-secretase in buoyant membranes (Wahrle *et al.* 2002). The functional consequences of the localization of SPP in rafts, if any, are currently unknown and deserving of further study.

2.3.   Substrates and Function

The original identification of SPP arose from the search for the protease responsible for the generation of self-peptides presented by HLA-E (Weihofen *et al.* 2002) (Table 2). These self-peptides are derived by intramembrane cleavage of the signal peptide of MHC class I molecules in the ER (Lemberg and Martoglio 2002). Once generated, the peptides bind to HLA-E inducing its cell-surface expression. On the cell-surface it is thought that the presentation of such self peptides by HLA-E protects the cell from natural killer cell attack. Using a photoaffinity inhibitor approach, SPP was demonstrated to be the target of an inhibitor that blocked the cleavage and presentation of MHC class I signal peptides by HLA-E (Lemberg and Martoglio 2002). Thus, it has been proposed that one major role of SPP is to function to regulate normal immunologic surveillance (Lemberg *et al.* 2001; Wolfe and Kopan 2004). Interestingly, a more recent report suggest that SPP may play a role in dislocation from the ER (Loureiro *et al.* 2006). Dislocation is a term used to refer to the process in which unfolded or misfolded proteins in the ER are exported from the ER into the cytoplasm where they are degraded by the ubiquitin proteasome system. SPP has been shown to associate with a protein, US2, that plays an essential role in dislocation of MHC class I heavy chains (HC) (Loureiro *et al.* 2006). Knockdown of SPP by RNA silencing did increase levels of MHC class I HC suggesting a possible functional link with dislocation (Loureiro *et al.* 2006). Unfortunately, no data on the need for SPP mediated proteolysis in the dislocation pathway has been reported. Thus, although there is a tantalizing link between the proven function of SPP mediated proteolysis of Class I molecules and HLA-E loading of the Class I peptides and a potential role of SPP in US2 mediated MHC class HC dislocation, it is not clear whether there is a functional relationship between the two processes.

Table 2. Identified SPP substrates

Substrate	Products	Functions
Signal peptides of human MHC class I molecules	HLA-E epitope	Immune surveillance
Signal peptide of prolactin	Cytoplasmic portion of signal peptide	Calmodulin signalling? (enhancer of prolactin secretion?)
Hepatitis C and GB virus polyprotein	HCV core protein	Virus Assembly

Several viral proproteins are also known substrates of SPP. The HCV core protein is cleaved by SPP (McLauchlan *et al.* 2002; Okamoto *et al.* 2004; Majeau *et al.* 2005; Ait-Goughoulte *et al.* 2006; Dev *et al.* 2006; Hope *et al.* 2006; Vauloup-Fellous *et al.* 2006). During biosynthesis of viral proteins in HCV infected cells, the immature HCV core protein is transiently anchored in the ER membrane via a C-terminal, signal peptide-like sequence. Intramembrane proteolysis of the signal peptide in the HCV core protein by SPP promotes the final processing of core protein and its release from the ER membrane into the cytosol (Majeau *et al.* 2005; Ait-Goughoulte *et al.* 2006). Although it has been reported that SPP catalyzed cleavage of the HCV core protein is required for hepatitis C virus like particle assembly, another report suggests it is not required for virus budding but does destabilize the viral capsid (Vauloup-Fellous *et al.* 2006). More recently, SPP has been shown to be required for GB virus core protein processing, and in this case may be required for productive infection *in vivo* (Targett-Adams *et al.* 2006). As GB virus is the closest phylogenetic relative of HCV, such studies have implicated SPP as a potential target for anti-viral therapy (Targett-Adams *et al.* 2006).

Using a variety of artificial constructs or peptides consisting of various signal peptides and mutant versions of these peptides several groups have attempted to define the specificity of SPP cleavage (Lemberg and Martoglio 2002; Okamoto *et al.* 2002; Nyborg *et al.* 2004a). Except for showing that SPP cleavage appears to require "shedding" of the luminal portion of the substrate by signal peptide cleavage, the results of these studies are somewhat divergent. This may be attributable to the different methods of evaluating cleavage. One report evaluating cleavage of substrates produced by *in vitro* translation reactions demonstrated that helix desta-bilising residues are required for SPP cleavage and that the flanking sequences can affect the process (Lemberg and Martoglio 2002). However, a subsequent study showed that a reporter substrate based on a transmembrane domain sequence that was not cleaved by SPP following *in vitro* translation could be cleaved by SPP when transfected into cells (Nyborg *et al.* 2004a). In any case, the study of the specificity of SPP is in its infancy and additional studies will need to determine the spectrum of substrates cleaved by SPP and the sequence specificity, if any, of SPP cleavage. Indeed, specificity studies of presenilin dependent γ-secretase activity show that the intramembrane cleavage mediated by this enzyme exhibits little sequence specificity (Nyborg *et al.* 2006b).

To date knockout or siRNA mediated knockdown studies of SPP have only been performed in *Drosophila*, *C. elegans*, and Zebrafish (*Danio Rerio*) (Grigorenko *et al.* 2004; Casso *et al.* 2005; Krawitz *et al.* 2005). No mouse knockouts have been reported. These studies suggest that SPP and its homologs have important functional roles in development. In *C. elegans*, deficiency of ce-imp-2 (a SPP like gene) causes a severe developmental phenotype (Grigorenko *et al.* 2004). The effect of knockout of the two other *C. elegan*s SPP homologs was not reported. *Drosophila* deficient in one of two SPP genes (CG11840) had defective trachea and died as larvae (Casso *et al.* 2005). In Zebrafish, when either the SPP or SPPL3 homologs were knocked down, an embryonic lethal phenotype was observed, with

a prominent effect on nervous system development noted (Krawitz *et al.* 2005) Furthermore, as discussed more extensively in a subsequent chapter, knockdown of the SPPL2b homolog resulted in a distinct developmental phenotype with an enlarged caudal vein (Krawitz *et al.* 2005). Based on these genetic studies it is clear that SPP and its homologs have important, and in some cases distinct, but essential, roles in normal embryonic development. The molecular details of these functional effects have not been elucidated. However, it seems unlikely that the SPP cleavage of substrates identified to date mediate the developmental phenotypes associated with SPP deficiency in these organisms.

The function of the closest homolog of SPP, SPPL3 remains unknown. Based on cleavage of several substrates and its cellular location it is possible that it has an overlapping function with SPP (Friedmann *et al.* 2004; Nyborg *et al.* 2006a). However, study of SPPL3 has been hampered by the difficulty with stable overexpression of this protein in mammalian cell culture (Nyborg, unpublished data). Again additional studies will be needed to understand the function of SPPL3 and other family members.

γ-Secretase cleavage may play dual roles in the cell. In some cases γ-secretase cleavage of substrates plays a role in signal transduction (Bray 2006). By catalyzing release of a cytoplasmic domain of the transmembrane substrate γ-secretase cleavage can regulate the release of the signalling domain of a transmembrane receptor (Landman and Kim 2004). In many cases, the release of the cytoplasmic domain allows it to translocate to the nucleus where it functions to modify transcription whether alone or in complex with other proteins (Fortini 2001; Okamoto *et al.* 2001; Marambaud *et al.* 2003; Murakami *et al.* 2003; Louvi and Artavanis-Tsakonas 2006). In other cases, γ-secretase cleavage has been shown to terminate a signal mediated via a transmembrane protein receptor (Taniguchi *et al.* 2003; Parent *et al.* 2005). Whether all cleavages of transmembrane protein substrates modulate signalling events is not known. γ-Secretase cleavage may also play a role more analogous to the proteasome, simply cleaving type 1 transmembrane proteins to generate smaller soluble fragments that can then be broken down by other proteolytic systems in the cell (Kopan and Ilagan 2004). Given the abundance of SPP, its location in the ER and its tentative link to ER dislocation, it is interesting to speculate that SPP and possibly SPPL3 may play a role in the clearance and turnover of normal and abnormal transmembrane proteins. By analogy, it is also tempting to speculate that the SPPL2 proteins may also play dual roles in signalling and protein turnover in other cellular compartments.

3.   SPPs AS THERAPETUIC TARGETS

The intense focus on PS/γ-secretase as a therapeutic target in Alzheimer's disease led to the development of extremely potent γ-secretase inhibitors (Dovey *et al.* 2000; Li *et al.* 2000; Seiffert *et al.* 2000; Lanz *et al.* 2003). Although some of these inhibitors are directed at the active site, others are not (Clarke *et al.* 2006). Such

studies provide proof of concept that the aspartyl I-CLiPs are, from a pharmaco-logic point of view, amenable drug targets; selective high affinity inhibitors with good pharmacokinetic properties can be identified that target γ-secretase *in vivo*. Unfortunately, due to mechanism based toxicity largely mediated by inhibition of Notch signaling (De Strooper *et al.* 1999), there are concerns that long-term admin-istration of a γ-secretase inhibitor will not be well-tolerated in humans (Hadland *et al.* 2001; Beher and Graham 2005; Siemers *et al.* 2005; van Es *et al.* 2005; Siemers *et al.* 2006).

As mentioned previously, initial inhibitor studies establish "proof of concept" that it is possible to selectively target the activities of SPP and γ-secretase. Based on their role in HCV core protein processing, human SPPs have been postulated to be a potential target for anti-HCV therapy. It is currently unknown whether SPP is a realistic clinical target with regards to HCV infection. Given the uncertainty of the normal functions of SPP, it is not clear whether treatment targeting it will be well tolerated. The possible functional overlap between SPP and SPPL3 suggests that it may be important and challenging to develop selective and non-selective SPP inhibitors which have the best combination of efficacy and safety.

It is also interesting to consider the clinical and biological consequences of a potential normal role of SPP in the processing of HLA-E epitopes. What are the consequences in the organism of inhibiting HLA-E presentation? Would it cause autoimmunity? Could altered SPP function play a role in autoimmune disease? Could manipulation of SPP function be a useful tool to modulate immune system function? Clearly, the study of SPP is in its infancy and the answers to such questions will require additional tools (e.g. selective potent *in vivo* inhibitors and SPP knockout mice) to help find answers to these interesting but speculative questions.

Recently, we have proposed that the SPP homologs present in several major human pathogens might represent novel drug targets (Nyborg *et al.* 2006a). Several important human pathogens have only a single SPP gene (see Table 2). In recent work we demonstrated that the plasmodium SPP (mSPP) does possess protease activity and that its activity can be inhibited by known inhibitors of SPP (Nyborg *et al.* 2006a). It seems possible, by analogy with more complex organisms, that mSPP could be a critical gene for malaria development and viability. If this is the case, then inhibition of mSPP may be lethal to the parasite. Given that the plasmodium SPP activity is inhibited by orally bioavailable small molecule γ-secretase inhibitors, it may be possible to develop drugs that selectively inhibit plasmodium SPP rather than human SPP or γ-secretase. If it can be shown that mSPP is a good drug target, similar techniques and reagents could be developed to target multiple human parasitic pathogens.

4.   CONCLUSION

SPPs represent a newly recognized and important family of proteases. Further study of SPPs will likely reveal novel physiologic and pathophysiologic functions in humans. Studies of SPP function in other organisms may also shed light on its

normal physiologic role and provide insight into whether SPP like proteins indeed represent novel therapeutic targets in certain parasitic disease. Moreover, given that SPP appears to function without co-factors, it is likely that further study of SPP and SPP homologs may provide substantial insight into the structure function relationship of aspartyl I-CLiPs.

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CHAPTER 3

GXGD-TYPE INTRAMEMBRANE PROTEASES

A family of novel aspartate proteases

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Abstract: Among the known intramembrane-cleaving proteases (I-CLiPs), the aspartate proteases are unique. Unlike I-CLiPs of the serine- and metalloprotease-type, which share their respective active site motifs with their classical counterparts, the aspartate protease I-CLiPs acquired a novel characteristic GxGD active site motif during evolution. These so-called GxGD-type proteases include the presenilin (PS), signal peptide peptidase (SPP), SPP-like protease (SPPL) families and the related type IV prepilin peptidase family, bacterial leader peptidases, which share the same active site motif, but which cleave their substrates directly at, rather than within, the membrane. PS, SPP and SPPLs adopt a similar, but inverted membrane topology with respect to their active site orientation. PS is the founding member of the GxGD-type I-CLiPs and has been identified as the catalytic subunit of γ-secretase. The major function of this protease complex appears to be the clearance of the remnants of a large number of type I membrane proteins that have undergone shedding of their ectodomains. For some substrates of γ-secretase, most prominently for the cell surface receptor Notch, γ-secretase cleavage is coupled with signaling by the release of a nuclear-targeted intracellular domain (ICD). In the case of Notch, the ICD functions in the nucleus as a key transcriptional regulator for cell differentiation in development and adulthood. In addition, γ-secretase is a pivotal enzyme in Alzheimer's disease (AD), responsible for the liberation of the AD-causing amyloid β-peptide from its precursor protein. SPP and SPPLs exert similar functions, which, however, use type II membrane proteins as substrates consistent with their opposite topologies compared to PS. Thus, the major function of SPP is likely to be to clear the ER membrane of signal peptides of secretory proteins, whereas SPPL2a and b have recently been shown to cleave tumor necrosis factor α to release an ICD that triggers interleukin-12 signaling. Despite the similarities in their overall biological functions, the major difference is that PS requires partner proteins for its proteolytic function, whereas SPP and probably also the SPPLs do not

Keywords: γ-Secretase, GxGD-type protease, intramembrane proteolysis, PS, SPP, SPPL

1.   INTRODUCTION

Because cleavage of a peptide bond requires water, researchers have argued for a long time that the hydrophobic environment of a membrane would probably be the last place in the cell, where proteolysis takes place. Intramembrane proteolysis has therefore been considered to be an obscure if not even an impossible process. The discovery of proteases capable of catalyzing peptide bond cleavage in the lipid bilayer of a membrane has demonstrated the existence of this process and opened a new and fascinating area in protease research. These so-called intramembrane cleaving proteases (I-CLiPs) are integral membrane proteins that span the membrane several times by α-helical transmembrane segments. So far, I-CLiPs of the metallo-, serine- and aspartate protease families have been identified (Wolfe and Kopan 2004). I-CLiPs of the cysteine type might exist as well, but have not been discovered yet. The active site residues of the I-CLiPs are typically part of the predicted transmembrane segments thus embedding them in the membrane or they are found in regions that are hydrophobic enough to dive into the membrane (Wolfe and Kopan 2004). I-CLiPs use single pass transmembrane proteins as substrates, that, depending on the I-CLiP, can be either in type I or type II orientation. Diverse functions can be attributed to the I-CLiPs, ranging from signal transduction via the liberation of extra- or intra-cellular signaling domains of the substrates to membrane protein processing and turnover (Weihofen and Martoglio 2003). Some, but not all I-CLiPs require that the substrate becomes first processed by another protease. This two-step cleavage mechanism, termed regulated intramembrane proteolysis (RIP) (Brown *et al.* 2000), is prototypically exemplified by the metalloprotease S2P (site two protease), the first I-CLiP, which was identified (Rawson *et al.* 1997). This protease contains a typical HExxH metalloprotease active site motif, which is predicted to be embedded in the membrane (Selkoe 2002). S2P cleaves SREBP (Sakai *et al.* 1996), a hairpin membrane protein, releasing its transcription factor domain from the membrane to regulate genes encoding the key enzymes of cholesterol biosynthesis (Goldstein *et al.* 2006). Substrate cleavage is strictly dependent on prior processing of SREBP by S1P (site one protease) (Sakai *et al.* 1996). The rhomboid proteases are I-CliPs of the serine protease type and contain a membrane-embedded GxSG serine protease motif (Urban *et al.* 2001). Rhomboids release extracellular ligands from their substrates, which are in type I membrane orientation, and in contrast to the cleavage by S2P, intramembrane cleavage by rhomboids occurs without initial cleavage by another protease (Freeman 2004). At present, the rhomboid proteases are the only I-CLiPs from which crystal structure information at atomic resolution has been obtained (Wang *et al.* 2006; Wu *et al.* 2006). The structure of the rhomboid GlpG suggests that rhomboids use a membrane-embedded catalytic serine-histidine diad to catalyze substrate hydrolysis (Wang *et al.* 2006). The two studies, however, leave the question open whether substrate hydrolysis takes place at the lateral surface of the protease (Wang *et al.* 2006) or in a pore-like cavity (Wu *et al.* 2006). The aspartate I-CLiPs are signified by a novel protease active-site motif. Unlike classical aspartate proteases, which contain a D(T/S)G(T/S) active site motif, these I-CLiPs do not share this motif but instead
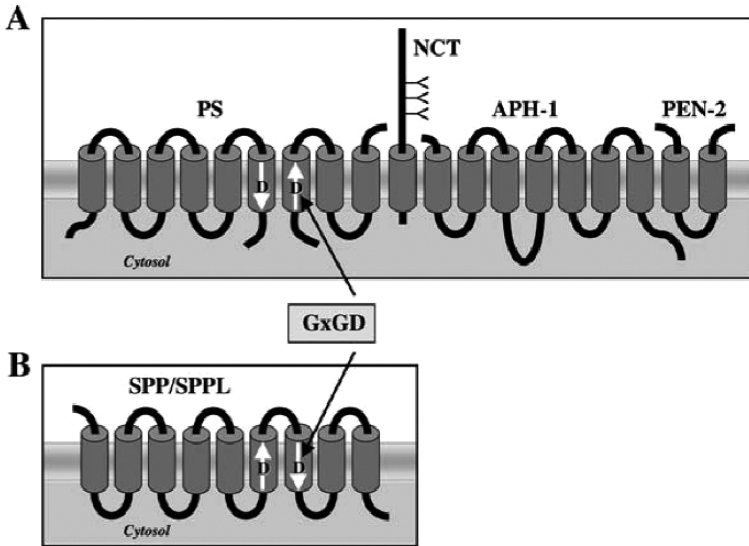
Figure 1. Intramembrane-cleaving GxGD-type proteases. (A) γ-Secretase with PS as catalytic subunit. (B) SPP and SPPLs. For details see text

are characterized by a novel GxGD active site motif, which comprises one of the two active site aspartates (Steiner *et al.* 2000) (Fig. 1). This motif was first identified in presenilin (the catalytic subunit of γ-secretase) (Fig. 1A) and the type IV prepilin peptidase (TFPP) (Steiner *et al.* 2000), a bacterial polytopic aspartate protease with its active site at the membrane / cytosol border (LaPointe and Taylor 2000). The subsequent discovery of additional families of aspartate protease I-CLiPs, the signal peptide peptidase (SPP) and SPP-like protease (SPPL) families (Fig. 1B) shows that all these proteases share the GxGD motif (Grigorenko *et al.* 2002; Ponting *et al.* 2002; Weihofen *et al.* 2002). Apparently, intramembrane proteolysis by an aspartate protease mechanism required evolution of an active site distinct from that of classical aspartate proteases.

2. INTRAMEMBRANE PROTEASES OF THE GXGD-TYPE

Presenilin (PS) is the prototype of the GxGD-type protease superfamily and has been identified as the catalytic subunit of γ-secretase (De Strooper *et al.* 1998; Wolfe *et al.* 1999; Esler *et al.* 2000; Li *et al.* 2000; Steiner *et al.* 2000) (Fig. 1A). This protease plays a key role in Alzheimer's disease (AD), a neurodegenerative disorder that is caused by the pathological deposition of the neurotoxic amyloid β-peptide (Aβ) in the brain of affected patients (Selkoe 2002). Aβ initiates a cascade of pathological events, the so-called amyloid-cascade, causing neuronal damage, progressive neuronal degeneration and cell death that ultimately results in dementia (Hardy and Selkoe 2002). Much of our current understanding of intramembrane

proteolysis has come from studies on the mechanism of Aβ generation. Aβ is generated by proteolytic processing of a larger type I transmembrane protein, the β-amyloid precursor protein (APP) by successive cleavages by β-secretase and γ-secretase (Haass 2004; Steiner 2004) (Fig. 2). β-Secretase cleavage removes the bulk of the large APP ectodomain first before γ-secretase attacks the C-terminal APP stub that is left in the membrane by intramembrane proteolysis. Another proteolytic cleavage, similar to that of β-secretase, in the APP ectodomain further proximal to its transmembrane domain (TMD) by α-secretase precludes the generation of Aβ. The resulting membrane stub is then turned over by γ-secretase cleavage. These amyloidogenic and non-amyloidogenic processing pathways of APP represent prototypic examples of RIP. In very rare cases AD is inherited as an autosomal dominant disease with an early disease onset. These familial forms of AD (FAD) are caused by mutations in the PS1 (accounting for the large majority of the FAD cases) and in the PS2 and APP genes, i.e. in the protease and its substrate
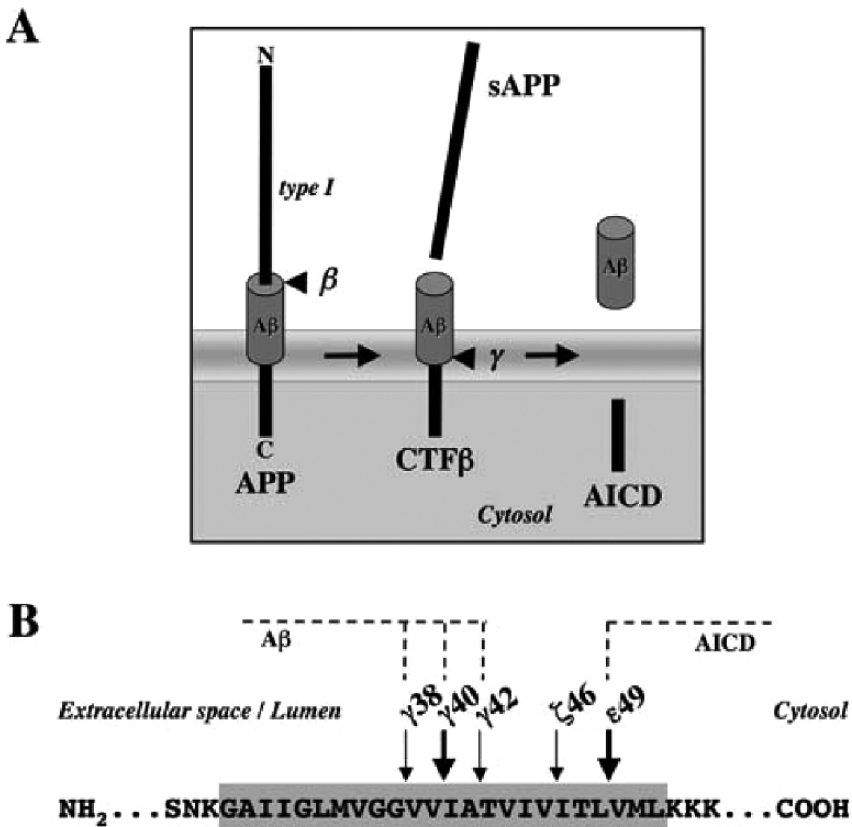

Figure 2. Processing of APP. (A) Amyloidogenic processing pathway. (B) Location of cleavage sites in the APP TMD. For details see text

(Hardy and Selkoe 2002). The mutations in PS, which adopts a nine-TMD topology (Henricson *et al.* 2005; Laudon *et al.* 2005; Oh and Turner 2005; Spasic *et al.* 2006), are found all over the protein, with a large number occurring in the TMDs and cause a shift in the cleavage precision of γ-secretase such that more of the highly neurotoxic and aggregation-prone Aβ42 species is produced (Scheuner *et al.* 1996; Citron *et al.* 1997). Most of the FAD mutations in APP locate near the γ-secretase cleavage site(s) and, like the FAD mutations in PS, cause an increase in Aβ42 generation (Scheuner *et al.* 1996). In addition, a double mutation has been identified at the β-secretase cleavage site. This double mutation causes a strong increase in the production of all Aβ species (Citron *et al.* 1992; Cai *et al.* 1993). Other mutations locate close to the cleavage site of α-secretase. These mutations do not change the production of Aβ or the precision of γ-secretase cleavage, but lead to mutant Aβ forms with enhanced aggregation properties (Hardy and Selkoe 2002).

The identification of PS as protease and catalytic component of the γ-secretase enzyme had been difficult because PS did not resemble any other known protease at the time of its discovery. Moreover, it even turned out that PS surprisingly does not exist as a full-length protein *in vivo* but is apparently cleaved into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) (Thinakaran *et al.* 1996) that form stable heterodimers (Capell *et al.* 1998; Thinakaran *et al.* 1998; Yu *et al.* 1998). A first clue that PS might be a protease probably even being identical with γ-secretase came from the seminal observation that embryonic fibroblast cells derived from PS1 knockout mice showed a strong reduction in Aβ generation (De Strooper *et al.* 1998). This deficiency in Aβ generation was accompanied by a massive accumulation of APP CTFs indicating that APP processing was affected at the γ-secretase cleavage step. Moreover, the residual γ-secretase activity was completely abolished when the PS2 gene was knocked out in addition (Herreman *et al.* 2000; Zhang *et al.* 2000). The same biochemical phenotype was observed with inhibitors that mimic the transition-state of an aspartate protease mechanism suggesting that γ-secretase is an aspartate protease (Wolfe *et al.* 1999; Shearman *et al.* 2000). If PS was therefore identical with γ-secretase it should contain two aspartate residues required for catalytic activity. Exactly this could be demonstrated in subsequent studies. Two highly conserved aspartates were identified in TMDs 6 and 7 of PS, which upon mutagenesis resulted in reduced Aβ generation with a concomitant accumulation of APP CTFs, i.e. again in an inhibition of γ-secretase activity (Steiner *et al.* 1999; Wolfe *et al.* 1999; Kimberly *et al.* 2000). Two other independent pieces of evidence strongly supported the idea that PS is an aspartate protease. First it could be shown that affinity-reagents based on aspartate protease transition-state analog inhibitors of γ-secretase capture PS in affinity-isolation experiments (Esler *et al.* 2000; Li *et al.* 2000). Second, the highly conserved region immediately preceding the proposed active site aspartate residue in TMD7 was not only functionally crucial for γ-secretase activity but strikingly resembled that of the TFPP (Steiner *et al.* 2000), a specialized bacterial leader peptide peptidase that had been shown to be an unprecedented novel polytopic membrane aspartate protease (LaPointe and Taylor 2000). Taken together with the knockout, mutagenesis and

inhibitor data, the additional identification of a highly conserved novel protease GxGD site conserved in PS strongly suggested that PS is an aspartate protease that acts as γ-secretase.

The finding that both PS and γ-secretase activity are associated with a high molecular weight complex suggested the existence of partner proteins of PS. These were identified as the integral membrane proteins Nicastrin (NCT) (Yu *et al.* 2000), a type I membrane glycoprotein, and the polytopic proteins APH-1 and PEN-2 (Francis *et al.* 2002), which together with PS form a functional γ-secretase complex (Lee *et al.* 2002; Steiner *et al.* 2002) (Fig. 1A). Reconstitution experiments in baker's yeast, an organism that lacks γ-secretase homologs, have demonstrated that the four subunits of γ-secretase complex are necessary and sufficient for γ-secretase activity (Edbauer *et al.* 2003). Similarly, overexpression of the four subunits in mammalian cells reconstitutes γ-secretase complex formation resulting in enhanced γ-secretase activity, which is not observed when only one subunit is overexpressed (Kim *et al.* 2003; Kimberly *et al.* 2003; Takasugi *et al.* 2003). In human cells, several distinct γ-secretase complexes exist, which are formed together with NCT and PEN-2 by either of the two PS homologs PS1 and PS2, and either of the two APH-1 homologs APH-1a (occuring in two splice forms) and APH-1b (Hebert *et al.* 2004; Shirotani *et al.* 2004). Assembly of these γ-secretase complexes is tightly regulated in a coordinated manner. The available data suggest a model for γ-secretase complex assembly where first NCT and APH-1 form an initial complex (LaVoie *et al.* 2003). In the next step of assembly PS joins this NCT/APH-1 assembly intermediate to form a ternary complex. Finally PEN-2 assembles to trigger PS endoproteolysis into its NTF and CTF (Takasugi *et al.* 2003). Following these assembly steps which take place at the endoplasmic reticulum (Kim *et al.* 2004; Capell *et al.* 2005), the γ-secretase complex traffics through the secretory pathway where it reaches its final destination in late compartments and the plasma membrane (Kaether *et al.* 2002; Chyung *et al.* 2005; Kaether *et al.* 2006).

The molecular weight of the γ-secretase complex has been estimated to be ~250–2000 kDa and apparently depends on the analytical method used (Li *et al.* 2000; Edbauer *et al.* 2002; Farmery *et al.* 2003; Kimberly *et al.* 2003). This suggests that the complex may adopt oligomeric forms or may contain additional regulatory subunits. Interestingly, two additional γ-secretase subunits, CD147 (Zhou *et al.* 2005) and TMP21 (Chen *et al.* 2006) that modulate the generation of Aβ have recently been reported. The electron-microscopic study of a ~300 kDa purified overexpressed γ-secretase complex revealed an almost spherical particle with two holes that might represent the exit sites for the cleavage products, and probably an interior chamber where hydrolysis may take place (Lazarov *et al.* 2006). Unfortunately, the resolution of the EM structure was too low to reveal details. Consistent with a water-containing cavity for proteolysis, several artificially introduced cysteines into TMDs 6 and 7 of PS1, were shown to be water-exposed suggesting that these TMDs of PS, which contain the active site aspartates, contribute to the proposed water-containing cavity in the γ-secretase complex (Sato *et al.* 2006; Tolia *et al.* 2006).
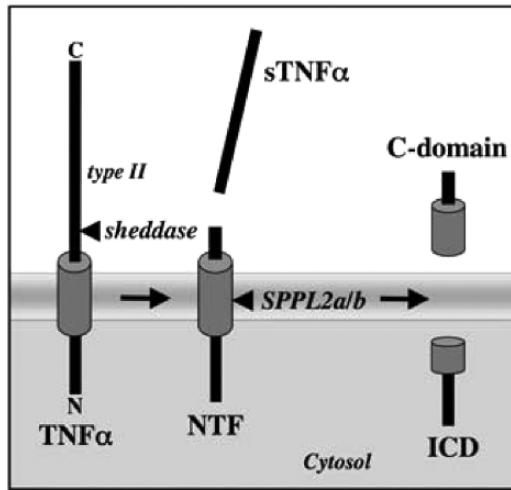
Apart from the AD-associated APP, a steadily increasing number of additional γ-secretase substrates have been identified (Kopan and Ilagan 2004) and as research continues many more are likely to be found. All substrates are type I membrane proteins that are processed in a very similar manner to APP. Like APP they are first shed in their ectodomain to generate CTFs in the membrane that are subsequently cleared by γ-secretase cleavage. One possible physiological function of γ-secretase is therefore the general turnover of membrane stubs of type I transmembrane proteins that have undergone ectodomain shedding (Kopan and Ilagan 2004). For about a handful of proteins, the intracellular domain (ICD) of the substrate that is released by γ-secretase cleavage translocates to the nucleus to regulate the expression of target genes. This nuclear signaling function, which is reminiscent of SREBP processing by S2P (Sakai *et al.* 1996), is most firmly established for the cell surface receptor Notch1 (Schroeter *et al.* 1998; Struhl and Adachi 1998), which probably represents the most important substrate of γ-secretase identified so far. The Notch1 ICD (NICD) released by γ-secretase cleavage (De Strooper *et al.* 1999), migrates to the nucleus to function as a crucial transcriptional activator of target genes required for cell differentiation during embryonic development and adulthood (Bray 2006). Genetic ablation of the PS1, APH-1a or NCT subunits of γ-secretase in mice causes Notch-like embryonic lethal phenotypes (Shen *et al.* 1997; Wong *et al.* 1997; Li *et al.* 2003; Li *et al.* 2003; Ma *et al.* 2005; Serneels *et al.* 2005). The Notch pathway is evolutionary highly conserved and Notch-deficient phenotypes are observed for mutants of the γ-secretase subunit homologs in *Drosophila* and *C. elegans* (Levitan and Greenwald 1995; Westlund *et al.* 1999; Goutte *et al.* 2000; Chung and Struhl 2001; Levitan *et al.* 2001; Francis *et al.* 2002; Goutte *et al.* 2002; Hu *et al.* 2002; Lopez-Schier and Johnston 2002). Notch-deficient phenotypes are also observed upon treatment of *Drosophila* or zebrafish embryos with γ-secretase inhibitors (Geling *et al.* 2002; Micchelli *et al.* 2003).

Another family of GxGD-type proteases is constituted by SPP. SPP has been identified as an I-CLiP of the ER capable of cleaving signal peptides of secretory proteins that are generated by signal peptidase (SP) suggesting that it functions to clear the ER membrane of otherwise accumulating signal peptides (Weihofen *et al.* 2002). In case of the major histocompatibility complex C (MHC) class I protein SPP-mediated processing of the signal peptide plays an important role in the human immune system. The combined cleavage of SP and SPP releases a highly conserved reporter peptide of MHC I that is presented at the cell-surface by the HLA-E protein (Lemberg *et al.* 2001). Exposure of this so-called HLA-E epitope reports proper biogenesis of MHC proteins to natural killer cells that would otherwise attack and destroy the HLA-E epitope presenting cells. In addition to these substrates SPP has been shown to cleave the hepatitis C virus polyprotein (McLauchlan *et al.* 2002) and the related GB virus B core protein (Targett-Adams *et al.* 2006). SPP therefore plays a crucial role as host-cell co-factor for the life cycle of these viruses. Apart from these known functions in humans, SPP has been found to be crucial for development in *C. elegans* (Grigorenko *et al.* 2004), *Drosophila* (Casso *et al.* 2005) and zebrafish (Krawitz *et al.* 2005).

SPPLs are the most closely related homologs of SPP and constitute additional families of candidate GxGD-type I-CLiPs (Grigorenko *et al.* 2002; Ponting *et al.* 2002; Weihofen *et al.* 2002). In human cells, four SPPLs divided in two subfamilies, SPPL2a, b, c and SPPL3 exist (Friedmann *et al.* 2004). Immunofluorescence microscopy studies, and assessment of their glycosylation status, indicate that SPPLs localize to distinct compartments of the secretory pathway (Krawitz *et al.* 2005; Friedmann *et al.* 2006). SPPL2a and b are both found in late compartments of the secretory pathway with SPPL2a in late endosomes and SPPL2b in endosomal/lysosomal compartments and/or the plasma membrane, whereas SPPL2c localizes to the ER. Localization to early secretory compartments, the ER and/or the Golgi has been shown for SPPL3. These data suggest that probably each compartment of the secretory pathway harbors its own SPP-type I-CLiP. SPP and SPPLs have a very similar membrane topology spanning the membrane nine times with their N-termini facing the exoplasm and their C-termini facing the cytosol (Friedmann *et al.* 2004). All of them, with the exception of SPPL3, are glycosylated (Friedmann *et al.* 2004; Krawitz *et al.* 2005). SPPL2a, b, and c contain N-terminal signal peptides that are removed upon integration of the proteins in the membrane (Friedmann *et al.* 2004). The so-called catalytic loop domain connecting TMDs 6 and 7, which contains the two active site aspartate residues, is localized to the exoplasm. Thus, the overall topology of SPP and SPPL is inverted compared to PS (Friedmann *et al.* 2004) (Fig. 1). This inverted topology is consistent with the reversed orientation of their respective substrates, which is type I for PS and type II for the SPP and SPPLs. Despite their inverted topology, the close relation of γ-secretase, SPP and SPPL2b with respect to their active sites is strongly supported by the observation that transition state analog-inhibitors of γ-secretase cross-inhibit SPP and SPPL proteases (Weihofen *et al.* 2003). Consistent with this observation, SPP can be affinity-isolated with an active site-directed γ-secretase inhibitor(s) (Nyborg *et al.* 2004; Sato *et al.* 2006). In addition, mutations of the first proline of a conserved PAL motif in the C-terminus of these proteases inhibit both γ-secretase and SPP activity and the affinity-capture with an active site-directed γ-secretase inhibitor. These data suggest that this critical region contributes to normal active site conformation of both proteases (Wang *et al.* 2006). In contrast to PS, SPP and SPPLs do not undergo endoproteolysis in their catalytic loop region (Weihofen *et al.* 2002; Friedmann *et al.* 2004). In addition, unlike PS, which together with NCT, APH-1 and PEN-2 forms γ-secretase complex(es), no other binding partners essential for activity have been identified for SPP and SPPLs suggesting that these proteases are not part of heteromeric protein complexes.

Information on the function of some of the SPPLs has recently been obtained. Downregulation of the SPPL homologs SPP and SPPL3 in zebrafish causes cell death in the central nervous system, whereas knockdown of SPPL2 expression resulted in an accumulation of erythrocytes in an enlarged caudal vein (Krawitz *et al.* 2005). Interestingly, the expression of active site aspartate mutants causes phenocopies (Krawitz *et al.* 2005). This demonstrates the specificity of the phenotypes observed and is consistent with the idea that these phenotypes are caused
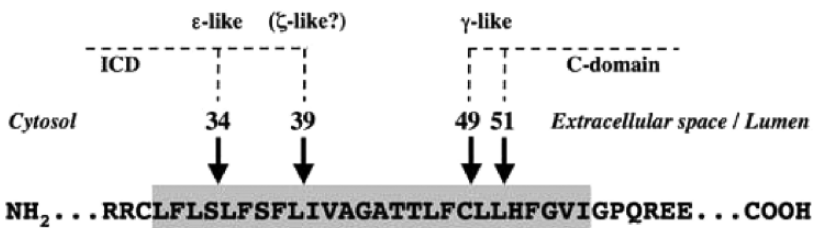
A

C

sTNFα

C-domain

type II

◄ sheddase

SPPL2a/b ►

N

TNFα   NTF   ICD

Cytosol

B

ε-like   (ζ-like?)   γ-like

ICD   C-domain

Cytosol   34   39   49 51   Extracellular space / Lumen

NH₂...RRCLFLSLFSFLIVAGATTLFCLLHFGVIGPQREE...COOH

Figure 3. Processing of TNFα by SPPLs. (A) Two-step processing pathway. (B) Location of cleavage sites in the TNFα TMD. For details see text

by the loss of proteolytic function. Very recently, human SPPL2a and b have been shown to cleave tumor necrosis factor α (TNFα) (Fluhrer *et al.* 2006) (Fig. 3). Cleavage of this substrate releases the ICD of TNFα, which acts as an activator of the pro-inflammatory cytokine interleukin-12 in activated human dendritic cells and implicates a function for SPPL2a and SPPL2b in the regulation of innate and adaptive immunity (Friedmann *et al.* 2006). Whether SPPLs have additional substrates is currently unknown.

3.   SUBSTRATE RECOGNITION OF GXGD-TYPE PROTEASES

The recognition of substrates by γ-secretase is mediated by a substrate receptor, which has been identified as NCT (Shah *et al.* 2005). This γ-secretase subunit, which has a large extracellular ectodomain (Yu *et al.* 2000), has been shown to recognize the free amino-terminus of the substrate (Shah *et al.* 2005) and apparently measures its length exposed to the extracellular/luminal space, which is typically

less than 30 amino acids. A study that investigated the influence of the length of the extracellularly exposed juxtamembrane region of model substrates on γ-secretase cleavage showed that the efficiency of substrate cleavage is inversely related to the length of the ectodomain. Substrates exposing more than 200–300 amino acids are not processed by γ-secretase, while substrates exposing less than 50 amino acids are cleaved with high efficiency (Struhl and Adachi 2000). Thus, to become a γ-secretase substrate, the bulk of the ectodomain has to be removed by sheddases first. After the initial recognition by NCT, the fate of the substrate, before it is eventually hydrolyzed at the active site is less clear. There is evidence that at least one additional binding site exists before the substrate enters the active site. Short helical peptides, 10 amino acids in length, designed to mimic the TMD of APP were shown to be potent inhibitors of γ-secretase, but did not target the γ-secretase active site directly (Kornilova et al. 2003). This site, referred to as docking site, to which the helical peptides bind has been identified in PS1 as its CTF (Kornilova et al. 2005). Because a slightly longer 13 amino acid helical peptide has partial access to the active site, it was concluded that the docking site is in very close proximity to the active site, probably with a distance only up to three amino acid residues (Kornilova et al. 2005). Consistent with these findings, a mutagenesis study aimed at the identification of critical amino acid residues of the active site domain in TMD6 and 7 of PS potentially involved in substrate recognition downstream of NCT implicated the GxGD motif in PS1 in substrate identification/selection of γ-secretase (Yamasaki et al. 2006). In this study, a critical amino acid was mapped at position x of the GxGD motif (L383 in PS1) that influences APP/Notch substrate selectivity (Yamasaki et al. 2006). These data indicate that the GxGD region plays a role beyond the catalytic function of γ-secretase in final substrate identification prior to processing and may be part of the proposed docking site. This site may serve as an entry port to the active site or contribute to such a function.

At present, the mode of substrate recognition by SPP and SPPL is not well understood. SPP can only cleave signal peptides of secretory proteins translocating to the ER that have been liberated by prior SP cleavage (Lemberg and Martoglio 2002). This requirement for an initiating cleavage is similar to the requirement of ectodomain shedding of γ-secretase substrates. Experiments using helical peptide inhibitors, analogous to those performed for γ-secretase, suggest that SPP probably also has a substrate docking site that is distinct from the active site (Sato et al. 2006). However, in contrast to γ-secretase, no substrate receptor has been identified and probably does not exist, because there is no evidence for heteromeric complex formation of these proteases except for SPPL2b that may have partner protein(s) (Nyborg et al. 2006). Moreover, another interesting observation has been made for SPPL2b, which could be found in physical contact with full-length TNFα (Fluhrer et al. 2006). This is different from the situation with γ-secretase, which cannot be specifically co-isolated with full-length substrates. This may suggest that recognition of type II membrane proteins as substrates of SPPL does not require recognition of their length.

4. SUBSTRATE CLEAVAGE OF GXGD-TYPE PROTEASES

Once the substrate has entered the active site, γ-secretase catalyzes the hydrolysis of the TMD of its substrates. The mode of this process has been most extensively studied for APP. The current model suggests that APP is cleaved in its TMD at two major sites, the γ-site, after residue 40 and at the ε-site, after residue 49 of the Aβ domain (amino acid residue numbering starting from aspartate 1 of the Aβ sequence) (Haass 2004; Steiner 2004) (Fig. 2B). These cleavages cause the liberation of the major 40 amino acid form of Aβ, Aβ40 (Dovey *et al.* 1993; Vigo-Pelfrey *et al.* 1993), into the extracellular space and the major 50 amino acid long form of AICD, AICD50, into the cytosol (Gu *et al.* 2001; Sastre *et al.* 2001; Yu *et al.* 2001; Weidemann *et al.* 2002). Additional cleavages occur to a minor extent at neighboring sites giving rise to the generation of small amounts of other Aβ species, Aβ37, Aβ38 and Aβ39, Aβ42 and Aβ43 (Wang *et al.* 1996; Wiltfang *et al.* 2001) and additional AICD species such as AICD51 and AICD48 (Gu *et al.* 2001; Yu *et al.* 2001). The proposed nine amino acid peptide intermediate was not identified, which however is consistent with recent data demonstrating that an additional cleavage after residue 46 at a novel site, termed ζ–site, occurs during γ-secretase-mediated cleavage of the APP TMD (Zhao *et al.* 2004; Qi-Takahara *et al.* 2005). The prevailing picture is that cleavage may start from the cytosolic border of the membrane at the ε-site and then proceed in a stepwise manner via the ζ-site to the γ-site by cleavage after every third amino acid on one side of the α-helical TMD (Qi-Takahara *et al.* 2005). However, although probably less likely, the other two possible directions of the cleavage, from γ to ζ to ε, or alternatively starting from the ζ-site and then proceeding from this site in two opposite directions to γ and ε cannot be fully excluded as, for example, APP mutants that block cleavage at either of the two major sites, γ and ε, have not been identified. This also suggests that γ-secretase does not recognize a specific sequence in the TMD of its substrates. γ-Secretase cleavage at two major, topologically distinct sites from those that the principal cleavage products are released has also been found for Notch1 and CD44 (Lammich *et al.* 2002; Okochi *et al.* 2002), whereas the identification of intermediate cleavage sites like the ζ-site in APP has not been reported for these substrates so far (Lammich *et al.* 2002; Okochi *et al.* 2002). The multiple intramembrane cuts conducted by γ-secretase, as exemplified by the γ-secretase cleavage of the APP TMD, is likely mechanistically required to ensure that the cleavage products can be completely liberated from the membrane. At present it is unclear, whether these multiple cleavages are executed by a single active site or by two or even three active sites within γ-secretase. Interestingly, evidence has been obtained that PS may be present in the γ-secretase complex as a dimer (Schroeter *et al.* 2003).

The cleavage-specificity of γ-secretase can be modulated by non-steroidal anti-inflammatory drugs (NSAIDs), pharmacological inhibitors of cyclooxygenase (COX). A subset of these compounds causes an alteration of the cleavage specificity at the γ-secretase site such that less of Aβ42 and more of Aβ38 are generated while the generation of Aβ40 is not affected (Weggen *et al.* 2001). Some NSAIDs

and NSAID-like compounds have an opposite effect and others increase Aβ42 generation (Kukar *et al.* 2005). The effects of NSAIDs on γ-secretase occur in a COX-independent fashion (Weggen *et al.* 2001) and although it is therefore likely that NSAIDs target γ-secretase directly, for which evidence has been provided (Takahashi *et al.* 2003; Weggen *et al.* 2003; Beher *et al.* 2004), the binding-site of NSAIDs has not been identified due to the lack of a suitable NSAID-based affinity-reagent.

SPP, in contrast to γ-secretase, cleaves signal peptides at one major central site (Weihofen *et al.* 2000), with, similar to γ-secretase, minor additional cleavages occurring at neighboring sites (Sato *et al.* 2006). SPP cleavage requires the presence of helix-bending or -breaking residues in the transmembrane region of signal peptides, probably to relax their α-helical structure in order to allow proteolytic attack (Lemberg and Martoglio 2002). In addition, efficient SPP cleavage requires the absence of charged residues in the transmembrane flanking regions (Lemberg and Martoglio 2002). Signal peptides lacking these requirements are poor substrates and escape SPP cleavage. Whether the active form of SPP is a monomer or a homodimer is unclear. Although specific labeling of the homodimeric form of SPP with a transition-state analogue inhibitor was observed, suggesting that the dimer is the active protease form, similar labeling-experiments in a follow-up study showed specific labeling of the SPP monomer and could thus not confirm the initial observation (Nyborg *et al.* 2004; Sato *et al.* 2006). Interestingly, it has recently been observed that NSAIDs also shift the cleavage specificity of SPP of a model signal peptide *in vitro* (Sato *et al.* 2006). Because the proteolytic activity of SPP, unlike γ-secretase, does not require partner proteins (Weihofen *et al.* 2002), these data may suggest that the NSAID binding site of γ-secretase lies in PS and that this binding site is structurally shared between these GxGD-type proteases.

SPPL2b cleavage of TNFα is mechanistically very similar to γ-secretase cleavage. Here, the TNFα-NTF, which results from ectodomain shedding by proteases of the ADAM family, is further processed by intramembrane proteolysis of SPPL2b at several sites (Fluhrer *et al.* 2006). These cleavages release the TNFα C-domain into the extracellular space and the TNFα ICD into the cytosol. The outer cleavage sites are located topologically analogous to the γ and ε-site of γ-secretase in relative proximity to the two membrane borders, whereas an additional cleavage site, which may be analogous to the ζ–site of γ-secretase is found in between (Fig. 3B). Whether the cleavage occurs in a stepwise manner in a certain direction is unknown at present.

5.   CONCLUSIONS

Considerable progress has been made in the past years in our understanding of intramembrane proteolysis, which not so long ago was a mysterious process. We now know the cellular function of many of the intramembrane proteases. Many interesting questions, however, remain to be answered. We still do not know how intramembrane proteolysis might work mechanistically or why γ-secretase, the most complex intramembrane protease, needs accessory proteins for its activity.

As research on the I-CLiPs continues, the focus will now more and more turn to obtaining structural information, which has recently been obtained for a rhomboid protease (Wang *et al.* 2006; Wu *et al.* 2006). Structural analyses will be very informative for the GxGD-type proteases as well, which although likely to be very difficult to obtain for the γ-secretase complex, may hopefully not be too remote for SPP and SPPLs.

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CHAPTER 4

RHOMBOID INTRAMEMBRANE SERINE PROTEASES

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Abstract: Intramembrane proteolysis catalyzed by rhomboid proteases plays key roles in such diverse cell communication events as receptor tyrosine kinase signalling during animal development, and quorum sensing during bacterial growth. In these contexts, rhomboid proteins act in the signal-sending cell to activate signal precursor proteins and initiate the signalling event. Recent biochemical advances have culminated in the first high-resolution crystal structures of an intramembrane protease, and a pure enzyme reconstitution system for studying rhomboid activity. Functional studies have expanded the cellular role of rhomboid proteins to broad biological processes, including host-cell invasion by malaria parasites, which is the first implication of these enzymes as possible therapeutic targets in human disease

Keywords: cell signaling, epidermal growth factor, protease, malaria, *Toxoplasma*, *Plasmodium*, invasion, rhomboid, intramembrane proteolysis, quorum sensing

1.   INTRODUCTION

Evolution of cellular life required the development of rapid and specific responses to cope with changing conditions. Many biochemical mechanisms that allow for rapid switches between cellular states have been characterized, but a previously unknown biochemical reaction, intramembrane proteolysis, has recently emerged as a common regulatory theme in widely diverse contexts. The conservation of intramembrane proteases across all kingdoms of life from Archaea to animals raises the intriguing possibility that they are among the most ancient regulatory enzymes of modern cells (Brown *et al.* 2000; Urban and Freeman 2002; Koonin *et al.* 2003).

Cells respond to more than just their physical environment: among the most complex series of changes that cells must decode are those that occur during the development of a multicellular organism from a single cell (Gerhart 1999). Evolution has adapted the full arsenal of regulatory mechanisms of eukaryotic cells to accomplish these feats, and studying developmental biology has served as a

fruitful approach for identifying the factors involved. In fact, the first intramembrane protease gene to be cloned was *rhomboid* (Bier *et al.* 1990), but the biochemical function of rhomboid as an intramembrane serine protease was among the last to be deciphered, over a decade later (Urban *et al.* 2001).

While rhomboid proteases clearly share defining biochemical characteristics with the other superfamilies of intramembrane proteases, they also have distinct properties that allow them to fulfill different biological roles (Urban and Freeman 2002). First, and most importantly, rhomboid proteins cleave near the outer leaflet of the membrane, and release domains to the outside of the cell. Other intramembrane proteases usually cleave near the inner leaflet of the membrane and release domains into the cytosol (often transcription factors). This difference has placed rhomboid protein function in the signal-sending cell, where they act as the signal-generating component that initiates cell signalling through cleavage of transmembrane ligand precursors.

Rhomboid proteins also differ in their biochemical mechanism in at least two fundamental ways (Urban *et al.* 2001). First, at the mechanistic level, rhomboid proteins are the only class of serine proteases known to catalyze intramembrane proteolysis; they evolved independently and are unrelated to other intramembrane proteases. Second, rhomboid proteins directly recognize their targets and cleave them as full-length proteins; they do not require a prior 'priming' cleavage, such as ectodomain shedding, to convert proteins into substrates (Urban and Freeman 2003). This characteristic raises questions both about how rhomboid proteases differ in achieving specificity and how they are regulated.

Although we still lack a sophisticated understanding of any form of intramembrane proteolysis, recent years have seen exciting advances in this newly-emerging field of study. I will focus on rhomboid proteins across evolution as a family (with the exception of the mitochondrial subclass that regulate membrane dynamics), and consider their functions in three broad sections. First, I will begin by describing the path of rhomboid protease discovery. Next, I will focus on our current understanding of their biochemical characteristics as a novel family of enzymes. Finally, I will consider the biological functions of rhomboid enzymes throughout evolution, where a diverse but small number of characterized examples indicate that rhomboid proteins are dedicated primarily to a signalling role, although this could reflect bias in the context in which they have been studied. A role for rhomboid enzymes in host-cell invasion by protozoan parasites is one exciting example of a recently discovered non-signalling function for this family of enzymes. This role has clear implications for human health, since these novel membrane enzymes may be potential therapeutic targets.

2.   RHOMBOID: THE PATH TO DISCOVERY OF AN INTRAMEMBRANE PROTEASE

2.1.   Abridged History of a Developmental Gene

Major mechanistic insights into the development of an organism from a single cell have been gained from genetic analysis of model organisms (Wolpert 1998). In the

late 1970s, the first screens to identify all genes required for embryonic patterning were initiated in the fruit-fly *Drosophila melanogaster* (Jurgens *et al.* 1984; Nusslein-Volhard *et al.* 1984). Among the ~126 genes that were identified was *rhomboid*, which was grouped with several other genes that had very similar embryonic phenotypes (most derive their names from a mis-shaped head skeleton) (Mayer and Nusslein-Volhard 1988). This *spitz*-group of embryonic genes consisted of *spitz*, *rhomboid*, *Star*, *pointed*, *single-minded*, and *sichel*. Actually, the first *rhomboid* mutation was isolated some years earlier and was called *veinlet* because it was a gene required for wing vein development (which occurs after embryonic development in *Drosophila*). The molecular biology revolution of the late 1980s resulted in the molecular identification of the *spitz* group genes: three membrane proteins and two transcription factors. The only protein to have a domain signature that might suggest a molecular function was Spitz, which contained an epidermal growth factor (EGF) domain in its predicted extracellular N-terminal domain (Rutledge *et al.* 1992). Were the Spitz-group proteins components of growth factor signalling?

Further genetic experiments ordered the Spitz-group proteins in the epidermal growth factor receptor (EGFR) signalling pathway, revealing that Spitz, Star and Rhomboid function in the signal-sending cell, upstream of the EGF receptor itself (Wasserman and Freeman 1997). Importantly, genetic removal of either Rhomboid or Star had the same consequences as removing Spitz, arguing that Spitz is inactive without Rhomboid or Star (Mayer and Nusslein-Volhard 1988; Guichard *et al.* 1999). Under physiological conditions, it was Rhomboid that was found to be the only component that is limiting for signalling, and its expression both prefigures signalling under normal conditions, and triggers it when ectopically induced (Bier *et al.* 1990). Thus, Rhomboid, a seven transmembrane protein expressed by the signal-sending cell, acts as the molecular 'on-off switch' for EGFR signalling, but how?

2.2. Spitz Signal Activation by Proteolysis

Despite the success of developmental genetics in identifying factors important for signalling and revealing their biological functions, this approach could not solve how Spitz is activated at the molecular level. Two biochemical systems were set up to study Spitz activation: a frog explant signalling assay (Bang and Kintner 2000), and direct biochemical examination of a tagged form of Spitz in *Drosophila* embryos by western analysis (Lee *et al.* 2001). Both approaches ultimately revealed that Rhomboid and Star are obligate components for the release of a soluble, truncated form of Spitz, but proved to be too complex to decipher the role of Rhomboid and Star. Cleavage of Spitz could be reconstituted in transfected cells in culture, which ultimately revealed the biochemical intricacies of how Spitz is activated.

Localization of Spitz, Star and Rhomboid revealed that Spitz resides in the endoplasmic reticulum (ER), and requires association with Star to exit the ER (Lee *et al.* 2001; Tsruya *et al.* 2002). Rhomboid is localized in the Golgi apparatus.

A pulse chase experiment revealed the order of events in Spitz activation (Lee *et al.* 2001); Spitz is translocated from the ER to the Golgi by Star, and there Spitz is cleaved. The cleaved form of Spitz undergoes extensive glycosylation (and it was later revealed palmitoylation (Miura *et al.* 2006)), and is released as a soluble ligand ready to activate EGFR signalling in neighbouring cells. These observations were also confirmed in developing *Drosophila* embryos (Lee *et al.* 2001; Tsruya *et al.* 2002).

Importantly, Spitz could be cleaved in the absence of Star, and the presence of Star had no effect on Spitz cleavage itself (Urban and Freeman 2003). Moreover, pulse-chase and experiments that targeted either Rhomboid or Spitz to different secretory pathway compartments indicated that Spitz does not need to be primed for cleavage by either being cleaved by another protease, or post-translationally modified (Lee *et al.* 2001; Urban *et al.* 2001). These observations collectively argued that Rhomboid alone was responsible for regulating Spitz proteolysis, but its molecular function remained unclear.

2.3.   Rhomboid is the Spitz Protease

Two models could be envisioned regarding how Rhomboid regulates Spitz cleavage: either Rhomboid activates an unknown protease to cleave Spitz, or Rhomboid itself was the Spitz protease. Since Spitz cleavage by Rhomboid occurred with similar efficiency in cell lines derived from different tissues and organisms, this suggested that Rhomboid itself might be the Spitz protease, rather than equivalent levels of a specific protease activity being supplied by all cells tested (Urban *et al.* 2001). The problem was that Rhomboid did not resemble any known protease (Bier *et al.* 1990), and it was not feasible to test Rhomboid for proteolytic activity directly due to the notorious difficulty in reconstituting activity with integral membrane proteins.

Instead, attention was focused on trying to understand how Rhomboid might function as a protease given what was known about proteolytic enzymes (Urban *et al.* 2001). It was possible to use the cell-based Spitz cleavage assay to search for putative catalytic residues in Rhomboid, which could reveal what type of protease Rhomboid would be, and thus indirectly address whether Rhomboid is a protease. This logic followed from decades of protease enzymology, which revealed that although many proteases evolved independently, they catalyze hydrolysis of peptide bonds using a small number of chemical mechanisms. Brian Hartley noticed this similarity, and in 1960 proposed a grouping of all proteases into what was at the time four catalytic mechanisms (Hartley 1960): serine proteases, aspartyl proteases, cysteine proteases, and metalloproteases (that often use histidines and a glutamate to coordinate zinc ions). Therefore, if Rhomboid was in fact a protease, then its active site residues should be essential for Spitz cleavage, and their identity might reveal a catalytic mechanism for Rhomboid.

Rhomboid proteins from various organisms contain ~18 residues that are conserved, and mutation of only 6 greatly reduced or completely abolished Spitz cleavage (Urban *et al.* 2001). The mutations did not alter the subcellular localization

of Rhomboid; all Rhomboid mutant proteins were localized in the Golgi apparatus, which indicated that they were not misfolded, because misfolded membrane proteins are retained in the ER. Two of these important residues, a tryptophan-arginine pair (WR), were located in the extracellular loop, while the remaining four were present in three different transmembrane domains (Fig. 1). Strikingly, the identity of three of these could correspond to a serine protease-like catalytic triad composed of a serine, histidine and asparagine, being contributed by three different transmembrane domains. Usually the third member of a catalytic triad in serine proteases is an aspartate (Blow *et al.* 1969; Fersht and Sperling 1973), but an asparagine had been found in a serine hydrolyase whose active site is embedded in the membrane (Snijder *et al.* 1999). Asparagines are also common in catalytic triads of cysteine proteases (Vernet *et al.* 1995).

The final essential residue, a conserved glycine, was located two residues upstream of the putative active serine. The second feature of a serine protease is
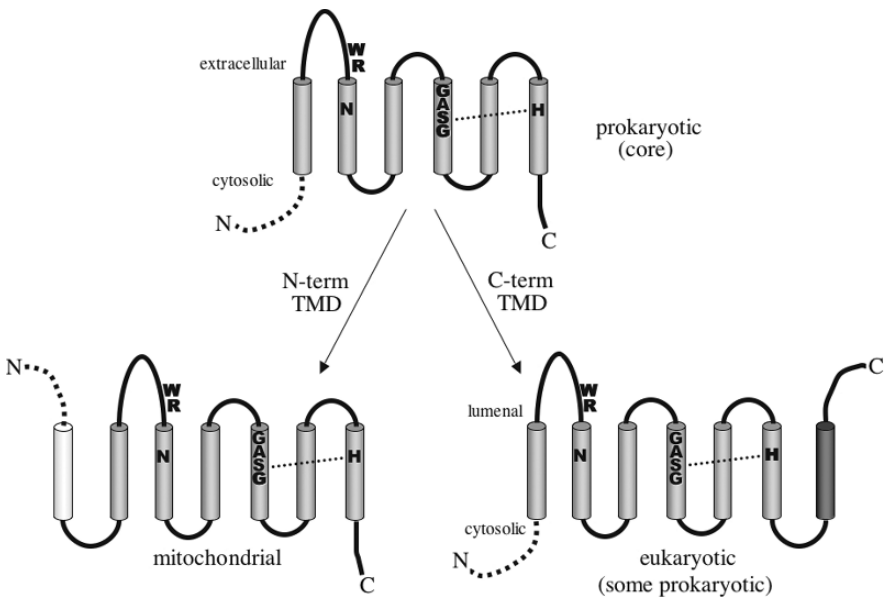

Figure 1. General topology of rhomboid proteins. The 'core' rhomboid domain consists of 6 transmembrane domains (TMD), which are depicted as grey cylinders. The most divergent region is the N terminal extension, which varies greatly in sequence and length (dashed line). Topology of the *Escherichia coli* and *Drosophila* proteins has been determined experimentally; the N-terminus is cytosolic for both proteins, while the C-terminus of the *Drosophila* rhomboid is in the Golgi lumen. The topology of the mitochondrial rhomboid proteins has not been resolved. Eukaryotic rhomboid proteins generally have an added TMD either N-terminal to the core (in white, left) as in the mitochondrial subclass, or added C-terminally (in black, right). Residues important for activity, as well as the conserved GASG sequence motif surrounding the active serine, are depicted. Only the S, H, and R have been found to be essential for activity in all rhomboid proteins tested. The hydrogen bond between the active serine and histidine catalytic pair is depicted with a dashed line

an oxyanion-stabilizing pocket, which binds the negatively charged oxygen atom that is generated in the transition state (Fersht 1973; Wilmouth *et al.* 2001). This oxyanion is often stabilized by binding to the backbone nitrogen of a glycine two residues upstream of the active serine. Alternatively, the amide of an asparagine has also been found to contribute to oxyanion binding in some proteases and could be provided by the essential asparagine in Rhomboid. However, the glycine is also located in a conserved GASGG motif, which is the only conserved sequence motif in rhomboid proteins (although the identity of the second and last two residues are not always necessarily A and GG in more divergent rhomboid proteins). This is strikingly similar to the GDSGG motif that surrounds the active serine in dozens of classical serine proteases (Blow *et al.* 1969), and implies that the glycine functions in oxyanion stabilization. Thus, the model was proposed that Rhomboid is itself the Spitz protease, and is a novel intramembrane serine protease (Urban *et al.* 2001).

The cell-based cleavage assay was further used to test some immediate predictions of this model. Accordingly, it was found that Spitz was the first growth factor known to be cleaved within its transmembrane domain, at a region that corresponded to the putative depth of the proposed catalytic triad within the membrane bilayer (Urban *et al.* 2001). Furthermore, this cleavage was sensitive to only a subset of serine protease inhibitors. These observations provided satisfying initial support for this speculative model.

2.4.   A Conserved Family of Intramembrane Serine Proteases

While developmental regulators are rarely conserved outside the animal kingdom, genome sequencing projects revealed that rhomboid proteins are conserved in all kingdoms of life, from bacteria to man (Wasserman *et al.* 2000; Koonin *et al.* 2003). In fact, rhomboid proteins may be the most widely-conserved family of membrane proteins currently known. However, rhomboid proteins are a divergent family that generally share only ~5% sequence identity as a group in their conserved core of six transmembrane domains (Urban *et al.* 2002b; Koonin *et al.* 2003). Further diversity is generated by the addition in eukaryotes of a transmembrane helix either N-terminally to the core, as occurs in the PARL family of mitochondrial rhomboid proteases, or C-terminally, as in most eukaryotic rhomboid enzymes (Fig. 1). Highly divergent soluble domains added to the termini add further diversity. The sequence divergence of rhomboid proteins, and their presence in bacteria and archaea, initially implied that the divergent prokaryotic forms may have functions unrelated to the *Drosophila* paradigm. Were divergent rhomboid proteins like those from bacteria also intramembrane proteases, or did these proteins have a more primordial function, such as membrane transport?

The functions, and therefore any putative substrates, for prokaryotic rhomboid enzymes are not known. Instead, eight different prokaryotic rhomboid proteins were tested for their ability to catalyze cleavage of Spitz in transfected mammalian cells (Urban *et al.* 2002b). Five of eight were found to cleave Spitz using the same putative active site serine and histidine residues as *Drosophila* Rhomboid-1

(although the asparagine was dispensable for activity in several rhomboid enzymes, including those from *E. coli*, *B. subtilis* and humans). This implied that many if not most prokaryotic forms were indeed also intramembrane proteases with similar properties.

Early observations therefore indicated that rhomboid proteins are a very widely-conserved family of intramembrane serine proteases. This was exciting on two levels: serine proteases are perhaps the best studied of all enzymes, yet rhomboid appeared to be a serine protease whose predicted function of catalyzing hydrolysis within membranes was heretical. Secondly, rhomboid proteins are among very few animal developmental regulators that are conserved in other kingdoms, arguing that they may be among the most ancient enzymes of modern cells, although almost nothing was known about their biological roles. Recent advances have made significant progress in these two important areas.

3.   RHOMBOID PROTEASE BIOCHEMISTRY

3.1.   Substrate Specificity of Rhomboid Proteases

Understanding how rhomboid proteins recognize and handle substrates is both valuable for elucidating their cellular roles (see section 4), and has added important insight into how hydrolysis of peptide bonds occurs within membrane bilayers. The first relevant biological context for the importance of specificity was how Rhomboid recognizes Spitz during *Drosophila* development. In fact, Rhomboid is the signal-generating component of EGFR signalling and must therefore cleave Spitz and not the thousands of other transmembrane domains that it encounters in the cell. Accordingly, Rhomboid cleaves Spitz, but not other type I transmembrane proteins such as the EGF receptor, the Notch receptor ligand Delta, and even Transforming Growth Factor alpha (TGFα), the human homolog of Spitz (Urban and Freeman 2003). A mapping study whereby chimeras between TGFα and Spitz were generated in order to identify the basis for Rhomboid-1 specificity revealed that Rhomboid recognizes the top seven residues of the Spitz transmembrane domain alone (Fig. 2). This 'substrate motif', comprised of the sequence ASIASGA, was both necessary and also sufficient, because placing just these seven amino acids into TGFα and Delta converted them into potent substrates for Rhomboid (Urban and Freeman 2003).

Strikingly, many rhomboid proteases from diverse organisms that are able to cleave Spitz are also dependent on substrate residues in the Spitz transmembrane domain, suggesting that substrate specificity for at least one subclass of rhomboid enzymes is conserved throughout evolution (Urban and Freeman 2003). But it should be noted that other rhomboid substrates exist that do not obviously resemble Spitz, and the basis for their cleavage is not understood (Urban *et al.* 2002a). Moreover, several rhomboid proteins exist that do not cleave Spitz, and at least in one case this was recently found to be due to different substrate specificity (Baker *et al.* 2006). Still, a large subclass of rhomboid proteins conform to these
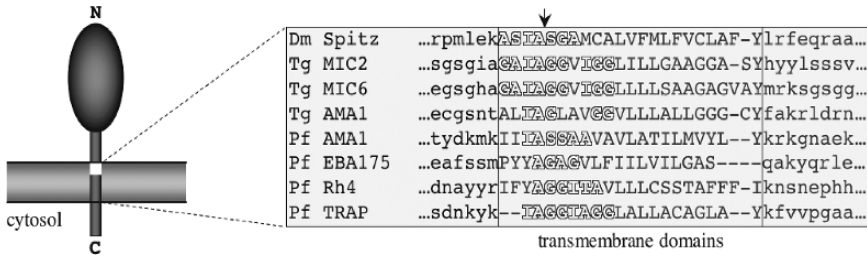
Figure 2. Substrate specificity of rhomboid proteases. Rhomboid proteases from a wide range of organisms have been shown to recognize the top 7 residues of the Spitz transmembrane domain (on left depicted in white). This substrate motif is composed of helix-relaxing residues such as glycines, and is conserved in adhesins from apicomplexan parasites (outlined letters on right). Only one protein of each adhesin family is shown for clarity, and other less characterized rhomboid substrates have been omitted. The natural cleavage site (designated by the arrow) usually occurs following an alanine residue, and has been determined for TgMIC2, TgMIC6, TgAMA1, PfAMA1 and PfEBA175. Transmembrane sequences are in uppercase lettering and boxed, but the exact site of membrane junctions are difficult to predict accurately. Dm, *Drosophila melanogaster*, Tg, *Toxoplasma gondii*, Pf, *Plasmodium falciparum*

rules, and deciphering the basis of Spitz targeting further revealed insights into how intramembrane proteolysis is achieved mechanistically.

Extensive mutagenesis of the Spitz substrate motif revealed that helix-destabilizing residues, especially a GA residue pair, are the key feature of this region (Urban and Freeman 2003). For example, replacing the glycine of the GA motif with a leucine abrogated cleavage. However, replacing it with proline, which has a similar side-chain but is known as a helix-breaker because its side-chain is covalently bonded to the preceding backbone nitrogen, allowed efficient cleavage. These types of experiments collectively support the notion that it is not the side-chain, but rather its effect on the secondary structure of the transmembrane segment, that is the key feature of the Spitz motif (Urban and Freeman 2003). This distinct feature, together with the general topology of the substrates being type I proteins (i.e. N termini extracellular; Fig. 2) and possible indirect effects such as steric hindrance of extracellular or cytoplasmic domains of putative substrates, make rhomboid proteins highly specific enzymes.

Interestingly, the apparent dependence of rhomboid proteins solely on helix-relaxing residues implies that substrates are not recognized by a specific physical interaction. Instead, the ability to unwind the helix is important for cleavage, and suggests that perhaps extension of the substrate into the active site of the enzyme may determine whether cleavage occurs, but these hypotheses await direct biophysical corroboration.

3.2.   *In Vitro* Reconstitution of Rhomboid Activity

The clear limitation that precluded the biochemical analysis of rhomboid proteins was the lack of a cell-free system that could recapitulate intramembrane proteolysis

in vitro. Indeed, the obvious direct test of the model that Rhomboid is a protease is to purify both putative enzyme and substrate and demonstrate Spitz proteolysis *in vitro*. The formidable challenge of reconstituting activity with pure integral membrane proteins hindered this approach initially, but having learned more about the unique properties of these enzymes and the defining features of their substrates allowed a more rational attempt.

Diverse rhomboid proteins that had been confirmed to function as intramembrane proteases in the cell-based system were tested for expression in recombinant form in *E. coli* (Lemberg *et al.* 2005; Maegawa *et al.* 2005; Urban and Wolfe 2005). Several rhomboid proteins could be expressed at high levels, and these were good candidates for subsequent purification. As with many membrane proteins, the experimental conditions had to be determined empirically. Stability and activity was found to rely primarily on using an appropriate detergent, with dodecyl-β-D-maltoside (DDM) being the best of the 10 detergents tested for rhomboid proteins (Urban and Wolfe 2005). Generally, non-ionic alkyl glucoside detergents with carbon chain lengths of 9 (nonyl glucoside) or longer are most conducive for activity.

Multiple rhomboid proteins can be purified to apparent homogeneity in active form. The rhomboid proteins themselves are very different from each other at the sequence level and were derived from incredibly different organisms: from *E. coli*, a model Gram negative bacterium, *B. subtilis*, a model Gram positive soil-dwelling bacterium, *Providencia stuartii*, a Gram negative human pathogen, *Aquifex aeolicus*, one of the most hyperthermophilic bacteria known that grows at ∼96°C, and from an animal, *Drosophila*. The reconstitution system is of course only as good as the least pure component added, thus the purity of the substrates was also a critical factor. Two general approaches have been used: generating a radiolabelled peptide substrate in a transcription-translation extract (Lemberg *et al.* 2005), and recombinant expression and purification of substrate proteins (Maegawa *et al.* 2005; Urban and Wolfe 2005). The recombinant Spitz-based substrate was adapted from a C-terminal fragment of amyloid precursor protein that has served as an efficient substrate for γ-secretase, by placing the Spitz substrate motif in its transmembrane domain (Urban and Wolfe 2005). This allowed an analysis of substrate specificity *in vitro*: diverse pure rhomboid proteins can cleave this pure substrate *in vitro*, and do so specifically since they cannot cleave the substrate if the Spitz substrate motif is not present. The other pure substrate that has been developed is based on the second transmembrane domain of LacY from *E. coli*, which is also cleaved efficiently, although the basis for its cleavage or whether it can be cleaved by rhomboid proteins other than GlpG from *E. coli* is unclear (Maegawa *et al.* 2005). In both cases, cleavage is monitored by western analysis, since both proteins are epitope tagged. The catalytic efficiency of the system in general is high, with low amounts of enzyme (1–100 ng) being able to cleave 10–1000 fold molar excess of the substrate to completion. However, the reaction itself is predictably slow and requires extended incubation times to proceed to completion.

The most dramatic determinant of *in vitro* activity encountered so far is the type of phospholipids and detergents used in the assay. Several rhomboid proteases lack measurable activity in pure form, but regain strong activity when they are

reconstituted into specific membrane lipids (Urban and Wolfe 2005). Conversely, several rhomboid proteins remain strongly active in the detergent-solubilized state in pure form, and addition of lipids has mild, if any, stimulatory effects. Both phospholipid head groups and tails have effects on activity, with the outcome being particular to the enzyme studied. Other than the clear importance of the detergent and lipids used in the solubilization and reconstitution, respectively, activity does not rely on specific conditions; omitting ATP from the pure enzyme system (Urban and Wolfe 2005), or depleting ATP carried over from a transcription-translation system (Lemberg *et al.* 2005), does not reduce substrate cleavage, and adding ATP does not enhance proteolysis. Activity is robust in 0-250 mM salt and pH 6 to 8, and does not depend on divalent metal ions, consistent with the model that rhomboid proteins are not metalloproteases (Urban and Wolfe 2005).

Importantly, this reconstitution system also demonstrated that rhomboid proteins do not require any necessary cofactors or proteinaceous partners to catalyze intramembrane proteolysis, in contrast to γ-secretase, which is known to function as a complex between presenilin protein as the catalytic core and at least three other proteins (Edbauer *et al.* 2003; Kimberly *et al.* 2003). These biochemical reconstitution experiments provided the missing biochemical evidence that rhomboid proteins are themselves novel intramembrane serine proteases. This advance resulted in the acceptance of rhomboid proteins into the Enzyme Commission database as the only known family of intramembrane serine proteases (family EC 3.4.21.105).

3.3.   Architecture of a Rhomboid Protease

The advantage of the *in vitro* activity assay is that it both offers a precise method to study rhomboid catalysis, and provides high quantities of pure, active recombinant protein for biophysical analysis to probe the mechanism in detail. Indeed, the landmark of obtaining several high-resolution crystal structures of GlpG, the rhomboid protein from *E. coli*, has recently been achieved (Wang *et al.* 2006; Wu *et al.* 2006). The key to generating well-diffracting crystals turned out to be removing the N terminal cytosolic tail and using nonyl glucoside, the detergent with the shortest alkyl chain length that supports activity.

The basic architecture of the enzyme fits well with many ideas that had been proposed regarding how hydrolysis of peptide bonds occurs within the plane of the membrane (Urban *et al.* 2001; Urban and Freeman 2003; Lemberg *et al.* 2005; Urban and Wolfe 2005). The GlpG transmembrane domains form a proteinaceous ring to protect a central hydrophilic cavity (Fig. 3, lower panel). The structural scaffold for the protein appears to be derived mainly from the first three transmembrane helices, which are longer and traverse the membrane completely. Surprisingly, the large L1 loop connecting transmembrane domains 1 and 2 is also partially inserted into the membrane to plug a hole between transmembrane helices α1 and α3 (Fig. 3, upper panels). The conserved WR motif, which is also found in the derlin proteins that are important for retro-translocation of proteins from the ER (Lemberg *et al.* 2005), serves to reach from the depth of this submerged L1 loop to bond to outer regions
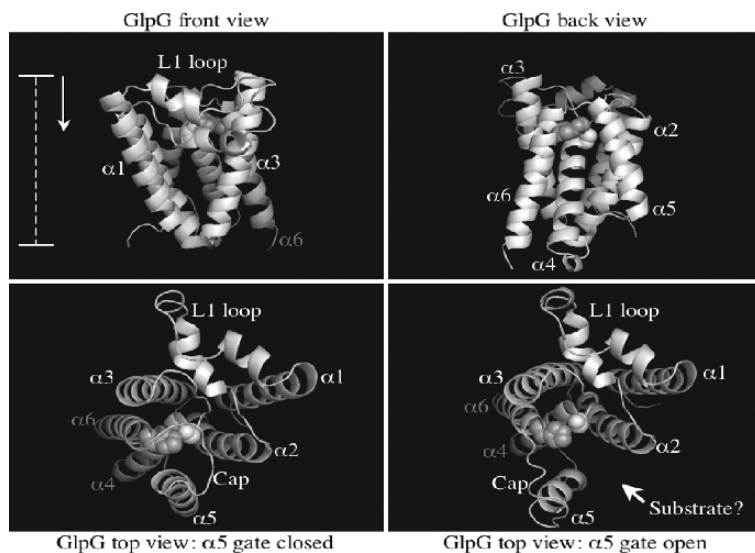
Figure 3. Crystal structures of GlpG, the rhomboid intramembrane protease from *E. coli*. The top two panels are viewed from the side, and the approximate thickness of the membrane is depicted by a dashed white line. Active site serine and histidine sidechains are in white and grey spheres, respectively. The front view illustrates the V-shaped gap formed by helices α1 and α3, which is 'plugged' by the L1 loop being partially submerged into the membrane. The back of the molecule illustrates the α4 helix that enters the core of the protease at an oblique angle, with the serine at the top of the helix, recessed ∼10 Angstroms (depicted by a white arrow) from the extracellular side (top). Top/extracellular view (bottom panels) illustrates two GlpG conformations observed in different crystal forms. The major difference is bending of transmembrane helix α5 outward laterally by ∼35 degrees, which also displaces the L5 loop (Cap). This creates an opening that could accommodate an incoming substrate helix. Note that it is mainly the top of α5 that rotates outward, leaving direct access to the active serine (which is on the side of the α4 helix facing α5 but opposite to L1) through an opening at the top of the protease ring. Images were created in Pymol from coordinates 2IC8 and 2NRF that have been deposited in the Protein Data Bank

of the loop. The central cavity of the enzyme itself is formed by a recessed helix α4 that enters the core from the bottom surface via an oblique angle, and stops in the center of the ring with the catalytic serine recessed ∼10 Angstroms from the extracellular surface. The top of the cavity is open and contains multiple water molecules in the structures. Loop 5 is thought to act as a 'cap', closing onto the cavity from the top and presumably restricting access of water molecules, although it is debatable whether water can actually be occluded from the cavity given its size and water content in the crystals.

The catalytic core of the enzyme is within the hydrophilic cavity and is comprised of a serine-histidine catalytic pair that interact via a strong hydrogen bond. The histidine ring is supported by stacking onto a neighbouring tyrosine ring, and through a hydrogen bond with an asparagine via a bridging water molecule. This extensive system supporting the histidine ring obviates the need for a third member

of a catalytic triad as is found in classical serine proteases (Urban *et al.* 2001; Lemberg *et al.* 2005). What is conspicuously missing is an oxyanion binding pocket, which was hypothesized to be formed by the backbone of the glycine two residues upstream of the catalytic serine, as occurs in other serine proteases. Mutation of this glycine to even an alanine abolishes all activity. However, in the current structures, this glycine is bonded to the L1 loop.

The limitation of the crystal structure is that it represents a static glimpse of an enzyme. Although the substrate would have to enter the inner hydrophilic cavity from the outer membrane environment, it is not clear how this occurs. One hypothesis is that the L1 loop is displaced, opening a gap between transmembrane helices α1 and α3 (Wang *et al.* 2006). This would allow the top region of the substrate transmembrane domain to slide into the active site via an opening left by the displaced L1 loop. It should be noted, however, that in this scenario the path of entry from the displaced L1 loop would mean that the catalytic serine in the active site faces away from the incoming substrate. Alternatively, the structure of GlpG in a different crystal form revealed that instead of the L1 loop, the short helix α5 on the other side of the molecule may be quite mobile (Wu *et al.* 2006); movement of α5 creates a lateral opening on the top of the protease ring (Fig. 3, lower panels), allowing substrate access to the central cavity. In this way, the substrate would enter the active site from the back of the ring, between transmembrane helices α5 and α2, and engage the catalytic serine face to face. Movement of the small helix α5 also results in movement of the extracellular Cap loop, presumably to facilitate further water entry into the central cavity.

It remains unclear which is the correct path of substrate entry or the location of the oxyanion-stabilizing pocket. But the general idea that a small gap near the outer leaflet of the membrane is opened on the protease, and that substrate entry requires helix-breaking residues in the substrate are a common feature of current models. Both models thus fit well with the substrate specificity data that predicted only the top of the substrate transmembrane domain determines whether cleavage occurs, and this region is comprised of helix-relaxing residues (Urban and Freeman 2003). In this way, many diverse transmembrane segments could dock onto rhomboid, but only the small number of proteins that contain flexible helices could unwind into the active site to be cleaved. The cleavage site in rhomboid substrates have been mapped directly in only a few contexts, most of which are in parasite adhesin molecules (see section 4). In all cases examined, cleavage was found to occur within the substrate motif but at the very top of the transmembrane region, and most after an alanine residue (Urban *et al.* 2001; Opitz *et al.* 2002; Zhou *et al.* 2004; Howell *et al.* 2005; Maegawa *et al.* 2005; O'Donnell *et al.* 2006). Cleavage does not occur directly at the helix-relaxing residues but rather further up, which is consistent with a model whereby the top region of the substrates is being unwound into the active site. However, further work is required to clarify the relationship between helix-relaxing residues and cleavage sites.

Additional structures of rhomboid enzymes in different conformations is also needed to decipher the steps in the reaction pathway, although care must be exercised to distinguish between active open conformations and partially disor-

dered forms that are not functionally relevant. Moreover, analysis of rhomboid enzymes from eukaryotes that have additional transmembrane domains and different loops will be a challenge in understanding the function of different forms of rhomboid proteases. Correlating structure with function through mutagenesis and analysis in the reconstitution assay will be valuable in differentiating between models.

3.4.   Regulation of Rhomboid Intramembrane Proteolysis

Intramembrane proteolysis by rhomboid proteases is a new paradigm in protease function, and little is known about how rhomboid activity is regulated. Proteases are usually precisely controlled enzymes, with significant regulation occurring at the post-translational level, through the production of proenzymes, endogenous proteinaceous inhibitors (serpins), and direct modulation of enzyme catalysis by protein modification. Conversely, there are two main modes of regulation for rhomboid proteins; regulation by transcribing the *rhomboid* gene during development (Bier *et al.* 1990), and by subcellular segregation of substrate and rhomboid protease, as in the case of Spitz being confined to the ER in the absence of Star (Lee *et al.* 2001). This is also similar to the physical segregation of parasite adhesin proteins from rhomboid enzymes prior to cleavage at the end of invasion (see section 4). For the case of *Drosophila* Rhomboid, these two modes are probably the major routes for regulating Spitz cleavage, but this is unlikely to be the case for all rhomboid enzymes.

The *in vitro* reconstitution assay has revealed that several rhomboid enzymes could potentially be regulated at the post-translational level by membrane environments (Urban and Wolfe 2005). Several rhomboid enzymes display specific membrane phospholipid requirements for activity, while other rhomboid enzymes analyzed in parallel did not show this dependence, arguing that the observed effects may be specific. For example, *B. subtilis* YqgP was found to be highly active in choline containing lipid environments *in vitro*, and although choline is not thought to be a constituent of *B. subtilis* membranes, other endogenous modified lipids provide similar effects. Similarly, certain animal rhomboid enzymes were found to be highly active in thicker membrane environments, such as those at the plasma membrane, rather than the thinner membrane environments of the ER and Golgi. Thus, these enzymes may be held inactive until they reach the cell surface, but this has not been demonstrated directly.

Finally, it should be noted that other modes of regulation are possible, including through rhomboid protein oligomerization. γ-secretase and signal peptide peptidase are thought to function as dimers or oligomers (Schroeter *et al.* 2003; Nyborg *et al.* 2004), and oligomeric forms of rhomboid proteins can also be detected, but it is unclear whether they have any physiological significance. Several rhomboid proteins that lack catalytic residues have been found to be encoded in the genomes of many multicellular organisms. Whether these catalytically inactive proteins function to regulate the activity of active members is one intriguing possibility but remains unknown.

3.5.   Prospects for Rhomboid Protease Inhibitor Design

Selective protease inhibitors are invaluable tools for the study of enzyme mecha-
nisms, both at the structural level and the enzymological level. Although no selective
inhibitors of rhomboid proteases have yet been reported, important biochemical
tools are now available with which to mount more comprehensive attempts at
developing selective compounds. Importantly, the pure enzyme system affords the
ability to probe the inhibitor profile of rhomboid enzymes in detail, because cell
impermeable compounds can be tested, and because interfering cytotoxic effects
would not impede analysis.

   Recent probing of rhomboid activity with classical serine protease prototypes has
provided some initial information: rhomboid enzymes were found to be unusually
refractory to most serine inhibitor prototypes (Urban and Wolfe 2005). In fact, all
compounds directed against the serine nucleophile did not have any measurable
effect on substrate cleavage, including classical compounds like sulphonyl fluorides
(e.g. PMSF, AEBSF, APMSF) and phosphonates (e.g. DFP). Only one class of
inhibitors, the isocoumarins, proved effective against all rhomboid proteases tested,
with more reactive electrophilic analogs, including dichloroisocoumarin (DCI),
being more potent. These compounds are inhibitory by alkylating the histidine,
rather than the serine. While it is not clear why compounds directed against the
serine are ineffective while those that target the histidine are potent, it might suggest
that rhomboid proteases function differently from other serine proteases, and may
require a conformational change to adopt an active form (Urban and Wolfe 2005).

   Unfortunately, isocoumarins are unstable, highly reactive, and toxic compounds
that would be difficult to develop into selective inhibitors. Indeed, the paucity of
serine protease inhibitor prototypes that can be adapted to inhibit rhomboid proteases
selectively will make inhibitor design more of a challenge than first anticipated. An
attractive alternative to rational inhibitor design is screening of chemical compound
libraries in high throughput assays, although this might also present a challenge with
such membrane-embedded enzymes. It should be noted that in addition to aiding
biochemical analyses, developing selective rhomboid inhibitors also promises to
facilitate harnessing the power of chemical biology in deciphering the biological
function of rhomboid enzymes in diverse organisms in parallel. This is one very
promising area of investigation for the future.

4.   FUNCTION OF RHOMBOID PROTEINS: FROM SIGNALLING TO PATHOGEN INVASION

Rhomboid proteins may be the most widely-conserved membrane proteins currently
known (Koonin *et al.* 2003), with nearly all bacterial and archaeal species encoding
one or, more rarely, two or three rhomboid proteins. Larger rhomboid gene families
exist in eukaryotes, but are clearly divided into two groups: the PARL group (that
are localized to mitochondria), and the rhomboid group that have broad functions.
Despite this commonality among all kingdoms of life, remarkably little is known
about rhomboid function in organisms other than *Drosophila*. Recent advances have

started to reveal roles for rhomboid proteases in other organisms, and hinted that rhomboid function may be primarily, but not exclusively, focused on regulating cell signalling across evolution. Recent work has also uncovered important new non-signalling roles that have implications for human health.

4.1. Rhomboid Function in Invertebrate Animals: Regulating EGFR Signalling During Development

The original *rhomboid* gene is the prime regulator of *Drosophila* EGFR signalling in most developmental contexts, but careful analysis of EGFR signalling compared to the effects of null Rhomboid mutations highlighted a few exceptional instances in which removal of Rhomboid has milder effects than anticipated (Bier *et al.* 1990; Wasserman *et al.* 2000; Urban *et al.* 2004). Genome sequencing revealed that *rhomboid* exists as a seven-member family in *Drosophila*, and rigorous genetic analyses have now revealed the physiological roles of four of the seven *Drosophila* rhomboids. With the exception of the mitochondrial Rhomboid-7 (McQuibban *et al.* 2006), the other two currently characterized rhomboid homologs, Rhomboids 2 and 3, have both primary and supporting functions in initiating EGFR signalling. Compared to the role of the original Rhomboid (now called Rhomboid-1), the function of other non-mitochondrial rhomboid homologs can be classified into three categories.

In certain cases, it was found that Rhomboid-1 is not expressed in the tissue where EGFR is required, and another rhomboid homolog thus plays the primary function. For example, while Rhomboid-1 is the main rhomboid in embryogenesis and is not expressed in the early male and female germlines, Rhomboid-2 was found to be expressed in germline stem cells and functions to send a signal to the soma (Schulz *et al.* 2002). This signal triggers encapsulation of the germline stem cells by the somatic cells, which is required for the differentiation of germline cells into gametes.

In other cases, Rhomboid-1 is expressed in the developing tissue in question but null mutations result in very weak or no phenotypes. In these cases, another rhomboid was found to play the primary role. For example, Rhomboid-3 is expressed in the developing eye, where it fulfils the major role for a rhomboid (Wasserman *et al.* 2000), while Rhomboid-1 mutations result in no discernible phenotype (but it has a supporting role because a double mutation has a more severe phenotype than removing Rhomboid-3 alone). Strikingly, it was discovered that *rhomboid-3* is actually *roughoid*, a classical fly rough eye mutation that was isolated in the 1920s.

Thirdly, there are several instances where Rhomboid-1 clearly has the primary role, but its mutation does not fully result in a phenotype as severe as Spitz mutants. In these cases, other rhomboid homologs have a compensating role in activating EGFR signalling in the absence of Rhomboid-1. For example, removing Rhomboid-1 during embryogenesis results in considerably less cell death of ventral epidermal cells than removing Spitz (Urban *et al.* 2004). It was subsequently discovered that removing both Rhomboid-1 and Rhomboid-3 more fully resembled the Spitz

phenotype. However, Rhomboid-1 clearly plays the primary role here, because removing Rhomboid-3 by itself has no phenotype.

In conclusion, the main rhomboid protease in most developmental contexts is the original Rhomboid-1 protease that was uncovered in the historic embryonic screens. However, more recent analyses have revealed that rhomboid proteins collectively as a family, rather than as a single protease, function to satisfy all of the requirements for EGFR signalling during development. Mechanistically, this may reflect evolution by duplication and divergence, with the new rhomboid expression being targeted to a new tissue to activate EGFR signaling (since all of the other EGFR components are broadly expressed). Consistent with this model, Rhomboids 1, 2 and 3 are most similar to each other, are able to cleave all of the *Drosophila* transmembrane EGFR ligands (Urban *et al.* 2002a), and are arranged as a gene cluster on the tip of chromosome 3. Thus, at least several rhomboid proteins function as tissue-specific regulators to satisfy the > 60 contexts in which EGFR signalling is known to function during development. But it should also be noted that currently tested rhomboid proteins also have slightly different biochemical characteristics that might aid in fulfilling particular needs in different contexts (Urban *et al.* 2002a). Moreover, other uncharacterized rhomboid genes exist in flies, and although two contain the elements required for proteolytic activity, neither their expression patterns nor physiological functions are known. Therefore, it is too early to conclude definitively whether all *Drosophila* rhomboid proteins function only as EGFR signalling regulators.

Despite the prominence of rhomboid proteins in regulating EGFR signalling in *Drosophila*, their roles in other animals is surprisingly limited. The nematode worm *Caenorhabditis elegans* encodes five *rhomboid* genes, but mutation or RNAi of three homologs most closely related to *Drosophila* Rhomboid-1 resulted in no phenotype at all (Dutt *et al.* 2004). In contrast, mutating the EGF ligand LIN3 results in embryonic lethality (Hill and Sternberg 1992). A series of genetic interaction experiments revealed that ROM1 plays a supporting role in EGFR signalling during vulval development, a well-studied EGFR induction event. The anchor cell is known to send an EGF signal to the underlying hypodermis to induce their differentiation to make the vulva (Hill and Sternberg 1992). Surprisingly, this initial EGF signalling event is independent of ROMs, and probably relies on a different protease, but ROM1 was found to be a transcriptional target of EGF signalling in the hypodermal cells receiving high EGFR signalling. Based on these observations, a model was proposed that a second wave of LIN3 cleavage by ROM1 in hypodermal cells results in a positive feedback reinforcement of the initial signalling event. LIN3 exists in several isoforms, and only the longest form that inserts 15 residues upstream of the transmembrane domain is dependent on ROM1 for activation, although whether these residues facilitate activation by ROM1 directly or indirectly is unknown. Despite this supporting role in EGFR signalling during vulva development, ROM1 is widely expressed in somatic tissues yet a role in the vulva was noticed only in a sensitized vulva background, thus roles in other tissues remain possible. Moreover,

at least three other ROMs exist in worms that contain elements required for activity, yet their functions have not been explored.

4.2.   Function of Rhomboid in Vertebrate Animals

Although a role for rhomboid proteins in vertebrate EGFR signalling was suspected by analogy to *Drosophila*, current evidence challenges this notion. At least 6 rhomboid proteins (termed RHBDLs for rhomboid-like, (Pascall and Brown 1998)) are encoded in the mouse genome: RHBDLs 1, 2 and 3 (also termed RHBDL4 or Ventrhoid) have the elements known to be required for activity, RHBDLs 5 and 6 lack the active site serine and thus are not likely to be catalytic, and PARL is a rhomboid localized to the mitochondria. EGFR signalling in vertebrates has been studied intensively because of its importance in human cancer. Compelling biochemical and genetic evidence has implicated ADAM-type metalloproteases in EGF ligand activation by ectodomain shedding (Peschon *et al.* 1998; Sahin *et al.* 2004). In contrast, no genetic analyses have been reported for human or mouse non-mitochondrial rhomboid proteins, and biochemically none are able to process any of the EGF ligands that have been tested for cleavage (although a few EGF ligands have yet to be examined) (Lohi *et al.* 2004; Pascall and Brown 2004). Thus, RHBDLs are unlikely to function as activating proteases for EGF ligands.

Contrary to expectation, analysis of RHBDLs 5 and 6, which lack active site serines, indicated that they physically associate with EGF ligands and can produce wing phenotypes consistent with EGFR signalling when overexpressed in *Drosophila* (Nakagawa *et al.* 2005). Although the mechanistic basis for these phenotypes is unknown, and it is not clear if they result from the direct activation of EGFR signalling, this analysis does raise the possibility that RHBDLs may have functions beyond acting as intramembrane proteases. In conclusion, current evidence suggests rhomboid intramembrane proteases are not involved in EGFR signalling in mammals, although more limited supporting roles such as those observed in worms remain possible. Ultimately, RHBDL knockout analysis will be essential to reveal their physiological functions.

In addition to genetic analysis, searching for RHBDL substrates is a complementary approach to deciphering their biological roles. Of all of the RHBDLs examined, proteolytic activity could be demonstrated only for RHBDL2: RHBDL2 from fish, mice and humans are able to cleave Spitz, and display similar substrate specificity as *Drosophila* rhomboid proteases (Urban and Freeman 2003). This observation allowed bioinformatic searches to be conducted for proteins that contain the Spitz-like substrate motif in the top portion of their transmembrane domains (Fig. 2). Approximately 30 candidates were identified from a search of the mouse genome, and cleavage analysis by two groups identified two possible substrates. Pascall and Brown identified B-type ephrins as plausible substrates that were cleaved very efficiently (Pascall and Brown 2004). Ephrin receptors are the most abundant receptor tyrosine kinases in animals and are involved in many aspects

of nervous system development, including acting as repulsive signals during axon migration; thus cleavage by RHBDL2 might result in physical disengagement from the ephrin receptor to facilitate turning away from the repulsive signal. However, it should be noted that no physiological evidence exists to support this role, and that ADAM-type metalloproteases have also been implicated in ephrin processing. The second search identified thrombomodulin, a transmembrane glycoprotein that affects thrombin activity during blood clotting, as a possible RHBDL2 substrate (Lohi *et al.* 2004). While a circulating form of thrombomodulin is known to exist, it is not clear if this is released by proteolysis, or whether it has a functional role. The role of RHBDL2 in ephrin signalling, or blood clotting via thrombo-modulin cleavage, have not been corroborated by other approaches, and thus remain speculative.

A developmental function for one RHBDL, Ventrhoid/RHBDL4, is implied by its dynamic expression pattern during embryogenesis (Jaszai and Brand 2002). Ventrhoid is expressed in several ventral tissues, including the ventral neural tube, but it is not known which signalling pathway Ventrhoid activates, or indeed if it has a signalling function at all.

4.3.   Rhomboid Proteins in Other Kingdoms: An Ancient Signalling Mechanism?

Initially, a particularly confusing observation was the discovery of rhomboid proteins in other kingdoms of life, including plants and bacteria, because these kingdoms had not evolved EGFR signalling. In fact, plants and animals are thought to have evolved multicellularity after diverging from a common ancestor, and have thus evolved developmental signalling mechanisms independently (Meyerowitz 2002). Yet *Arabidopsis thaliana*, for example, contains over a dozen rhomboid genes. What is the role of rhomboid proteins in these kingdoms?

A preliminary analysis of two *Arabidopsis* rhomboid proteins indicated that one is able to cleave Spitz specifically, but not TGFα, while both rhomboid proteins were localized in the Golgi apparatus in cultured plant cells (Kanaoka *et al.* 2005). The very basic elements of rhomboid intramembrane proteolysis are thus conserved in plants. However, genetic knockouts of both proteins displayed no discernible phenotypes. Given that these two rhomboid proteins were found to be expressed in every tissue tested, the large number of rhomboid proteins and their apparent wide expression in plants might pose a significant barrier to genetic analysis because of the potential for redundancy of function. Plants also contain rhomboid proteins that are targeted to chloroplasts, and while their functions are also not clear, a role in cell signalling is unlikely.

Although almost all known bacteria contain at least one, and at times two or three rhomboid proteins, their functions have largely eluded study. In fact, rhomboid function in bacteria is currently understood at even the most basic level in only one organism, *Providencia stuartii*, a human pathogen of the

urinary tract. Many bacteria alter gene expression in response to differences in population size, and this regulation is accomplished by communication between cells, termed quorum sensing because the population is 'queried' (Waters and Bassler 2005). Signals released by bacteria accumulate as the population grows, and activate or repress genes appropriate for high versus low population size. A variety of small molecule autoinducers and their cognate signalling pathways have been discovered, but much remains unknown. Remarkably, two separate genetic screens aimed at identifying factors required for producing the quorum-sensing signal in *Providencia* identified AarA, a rhomboid homolog (Rather and Orosz 1994; Rather *et al.* 1999). At the time nothing was known about the molecular function of rhomboid, and the similarity with *Drosophila* rhomboid was so distant that it was not noticed until years later, but the similarity to signal production in *Drosophila* development, at least in superficial terms, is striking.

Despite this potentially exciting link, neither the signal activated by AarA, nor the molecular function of AarA were known. This was confounded by the fact that at the time Gram negative bacteria were thought to use acylated homoserine lactones and furanosyl boronate esters for quorum sensing, but not proteins (peptide quorum sensing signals generated from precursor proteins were thought to be limited to Gram positive bacteria) (Bassler 1999). But transgenic expression of AarA during *Drosophila* development partially rescued defects associated with Rhomboid-1 and Rhomboid-3 mutants, while expression of Rhomboid-1 in *Providencia* partially rescued signal production in *aarA* mutants (Gallio *et al.* 2002). An enzymatic analysis revealed that AarA is able to process all three *Drosophila* EGF ligands, and displayed similar substrate specificity to the *Drosophila* rhomboid proteases (Urban *et al.* 2002b). Therefore, these observations suggest that the signal-generating mechanism in *Providencia* and *Drosophila* are the same, and raise the intriguing possibility that this is the first signalling mechanism known to be conserved between bacteria and animals.

Whether the signalling role of rhomboid in *Providencia* is a common function or an exceptional case is not known. Notably, the *aarA* phenotype in *Providencia* is pleiotrophic (Rather *et al.* 1999), and thus AarA may have functions beyond quorum sensing. Recently rhomboid genetic knockouts have been made in several bacteria, including *E. coli* and *B. subtilis*. While the analysis in *E. coli* failed to reveal a phenotype despite testing 20 different metabolic conditions (Maegawa *et al.* 2005; Clemmer *et al.* 2006), the genetic null mutant of *B. subtilis* YqgP displays incomplete cell division resulting in a chaining phenotype (Mesak *et al.* 2004), which is reminiscent of the chaining defect also observed for *aarA* in *Providencia*. Thus, the role of rhomboid proteins in bacteria may also extend beyond quorum sensing.

4.4.   Rhomboid Proteases in Disease: Invasion of Host-Cells by Protozoan Parasites

Rhomboid proteins have only recently been implicated as possible therapeutic targets for the treatment of human disease. Protozoan parasites of the phylum Apicomplexa include *Toxoplasma gondii*, *Cryptosporidium* spp., and *Plasmodium* spp., the malaria parasites, and are among the most devastating blights globally (Greenwood and Mutabingwa 2002). Malaria alone affects over 10% of the world's population, claiming over a million lives each year, mainly children. *Toxoplasma gondii* causes toxoplasmosis, which remains a major cause of mortality in immuno-compromised patients, while *in utero* infection is a leading cause of neurologic birth defects (Carruthers 2002).

Apicomplexan pathogens are obligate intracellular parasites, and this dependence on an existence inside host cells has made understanding invasion mechanisms an important goal. Unlike other intracellular pathogens that achieve internalization by triggering endocytosis by the host, apicomplexans actively invade cells through an intricate process driven by a form of parasite motility (Sibley 2004). Videomicroscopy and biochemical studies have elucidated the basic steps in the invasion program.

Initial low-affinity attachment is mediated by GPI-linked surface antigens on any surface of the parasite, but high affinity interactions essential for invasion are established at the apical pole of the parasite (Dvorak *et al.* 1975) (Fig. 4). This interaction commits the parasite to invasion, and is established by secretion of transmembrane adhesins from specialized internal organelles called micronemes and rhoptries to the apical surface (Carruthers *et al.* 1999; Alexander *et al.* 2006). Adhesins are a diverse family of single-pass membrane proteins that exhibit tremendous variety in signalling and adhesive domains on their extracellular side. This variety is thought to facilitate invasion of different cell types through several different invasion receptors and pathways (Duraisingh *et al.* 2003). The adhesins engage host-cell receptors on the outside, and link to the cytoskeleton on the inside of the parasite, allowing translocation of the moving junction composed of adhesin-receptor complexes to the posterior of the parasite (Dvorak *et al.* 1975; Jewett and Sibley 2003). This provides the force to propel the parasite into the host cell and causes the invagination of the host plasma membrane into the nascent parasitophorous vacuole.

Proteolysis plays key roles during the invasion program, perhaps most prominently at its conclusion. Once the moving junction is translocated to the posterior of the parasite, it must be dissolved to allow successful internalization of the parasite within the parasitophorous vacuole, and sealing of the host-cell membrane. The irreversible moving junctions are known to be released by proteolysis of the adhesins by a parasite-encoded protease, but the identity of this enzyme, termed micronemal processing protease 1 (MPP1), eluded identification (Carruthers *et al.* 2000).

MPP1 is an unusual enzyme, because it was found to be inhibited by very few protease inhibitors, including DCI (Carruthers *et al.* 2000). Moreover, initial cleavage site mapping in one *Toxoplasma* adhesin suggested that cleavage might be occurring within the adhesin transmembrane domain (Opitz *et al.* 2002).
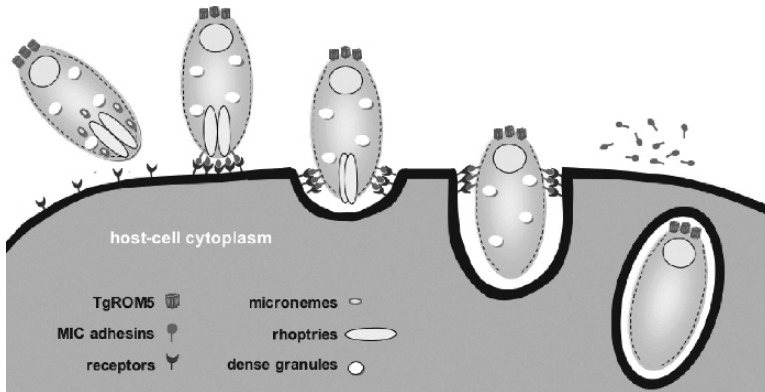
Figure 4. Invasion of host cells by apicomplexan parasites. Shown is a schematic model of *Toxoplasma* invasion. MIC and AMA1 adhesins are secreted from microneme organelles at the beginning of invasion, and engage host-cell receptors. The resulting moving junctions are motored to the posterior tip of the parasite, forcing the parasite into the host cell. Colocalization of TgROM5 with adhesins at the back of the parasite results in cleavage and disassembly of the moving junction, culminating in parasite internalization within the host cell, protected from the cytosol by the parasitophorous vacuole (in black)

Concurrently, an analysis of rhomboid protease substrate specificity identified the Spitz substrate motif as a key element in recognition of substrates by evolutionarily divergent rhomboid proteases, and a bioinformatic search for membrane proteins with a substrate motif in organisms other than *Drosophila* identified apicomplexan adhesins (Urban and Freeman 2003) (Fig. 2). Indeed, *Toxoplasma* adhesin transmembrane domains could be cleaved by rhomboid proteins from bacteria, *Drosophila* and humans. This observation led to the proposal that rhomboid proteases might be the elusive MPP1 activity (Urban and Freeman 2003).

Despite being a unicellular parasite, *Toxoplasma gondii* encodes six distinct rhomboid proteins (TgROMs) (Dowse and Soldati 2005). One of these, TgROM6, is predicted to be localized in the mitochondria, leaving five rhomboid proteins with all of the elements known to be required for catalysis as possible candidates for MPP1 during invasion. Activity analysis revealed that four of these are active proteases that share substrate specificity with other rhomboid enzymes for the substrate motif in Spitz (Brossier *et al.* 2005; Dowse 2005). However, adhesins are proteins with very large ectodomains, which can be refractory to intramembrane proteolysis, and only one *Toxoplasma* rhomboid protease, TgROM5, was able to catalyze processing of full-length adhesins, and cleavage was inhibited by DCI (Brossier *et al.* 2005). TgROM5 was also the only ROM found to be localized to the posterior surface of the parasite, the expected site of adhesin cleavage (Brossier *et al.* 2005; Dowse 2005); as with Spitz, the substrate is localized to a different part of the cell until cleavage is required. Two other adhesins have also been shown to be cleaved in their transmembrane domains, and all are substrates for TgROM5. Thus it was proposed that TgROM5 is primarily responsible for providing the

MPP1 activity essential for host-cell invasion, although it remains possible that other TgROMs also participate.

Plasmodia undergo a more complicated lifecycle with four invasive stages and over a dozen adhesins that have been implicated in the various stages of invasion, playing several distinct molecular roles (Soldati *et al.* 2004). Despite its importance, very little is known about adhesin processing during *Plasmodium* invasion. *Plasmodium falciparum*, the species that causes the lethal form of malaria, encodes 8 *rhomboid* genes (PfROMs), which are numbered according to their similarity to the *Toxoplasma* ROMs (Dowse and Soldati 2005). PfROMs 1, 3, 4 and 6 having direct orthologs in *Toxoplasma*, while *Plasmodium* also encodes four other rhomboid proteins, PfROMs 7, 8 and 9 of which have fewer transmembrane domains and larger loops, while PfROM10 lacks an active site serine and is not conserved in other *Plasmodium* species. Contrary to expectation, *Plasmodium falciparum* does not encode a direct homolog of TgROM5. Moreover, several families of *Plasmodium* adhesins contain aromatic residues, that are not conducive for cleavage by rhomboid proteases, within the top of their transmembrane domains. More recently, one adhesin that is cleaved by intramembrane proteolysis in *Toxoplasma* was found to be cleaved outside the membrane by a membrane-bound subtilisin-like protease (Howell *et al.* 2005). These early observations cast doubt on whether rhomboid enzymes play roles in *Plasmodium* invasion.

Further investigation revealed that EBA-175, a key adhesin known to be important for invasion of red blood cells, was cleaved in its transmembrane anchor during invasion (O'Donnell *et al.* 2006). An enzymatic analysis revealed that although *Plasmodium* does not encode a TgROM5 ortholog, its specificity is covered by two distinct rhomboid enzymes, namely PfROMs 1 and 4 (Baker *et al.* 2006). PfROM1 has the canonical specificity for Spitz-like substrates, while PfROM4 contains novel specificity that is able to process *Plasmodium* adhesins that contain aromatic residues, including EBA-175. TgROM5 is a 'dual specificity' protease, being able to cleave both Spitz-like and aromatic residue-containing adhesins. Together, PfROMs 1 and 4 were able to process all four families of *Plasmodium* adhesins, cleaving 13 of the 14 adhesins tested, and thus potentially broadening the role of rhomboid enzymes to all invasive stages of the malaria lifecycle, both in the human and mosquito hosts (Baker *et al.* 2006).

Importantly, different adhesins have been found to be expressed in different *Plasmodium* strains in the wild, correlating with the ability to invade different blood subtypes (Duraisingh *et al.* 2003). Moreover, genetic disruption of EBA-175 was found to result in upregulation of a different adhesin, with a concomitant switch in invasion pathway (Stubbs *et al.* 2005). Thus, while invasion of host cells proceeds through many diverse receptors and pathways, current evidence raises the possibility that most adhesins are cleaved by rhomboid proteases. This surprising commonality suggests that rhomboid-mediated cleavage might be a convergence point of several different invasion pathways, and suggests a possible point for therapeutic intervention. However, it is not known whether inhibiting rhomboid cleavage in parasites, either *Toxoplasma* or *Plasmodium*, would result in a block of

invasion, or whether the parasites have compensating mechanisms. One encouraging observation is that introducing a mutated form of EBA-175 that cannot be cleaved by PfROM4 did not support parasite growth, although the basis is unclear (O'Donnell *et al.* 2006). Inhibition of rhomboid proteases during invasion remains an important area for further exploration (when selective compounds are developed).

Finally, although a role for rhomboid enzymes in host-cell invasion by *Toxoplasma* and *Plasmodium* is supported by solid evidence, their possible roles in other aspects of the lifecycle have not been explored. Strikingly, *Toxoplasma* encodes TgROMs 1, 2, and 3, all of which are active proteases that are localized in different organelles, and are expressed at various stages of the lifecycle, but they do not appear to be involved in invasion (Brossier *et al.* 2005; Dowse *et al.* 2005). *Plasmodium* also encodes rhomboid enzymes whose functions have not yet been explored, although a role in cell communication in both parasite species remains an exciting but unsupported possibility.

5. CONCLUDING REMARKS

A remarkable amount of progress has been made in understanding rhomboid protein function in biochemical terms during the past 5 years – from the initial proposal that rhomboid proteins function as intramembrane proteases during development (Urban *et al.* 2001), to the recent first high-resolution crystal structures of a rhomboid protease (Wang *et al.* 2006; Wu *et al.* 2006). Yet it is striking that these advances have set the stage for studying these enzymes rather than solved their mechanism or biological roles. The high-resolution crystal structures of a rhomboid protease are a remarkable advance, and although the current structures are static views of what is likely to be a highly dynamic enzyme, they provide intriguing ideas to test regarding how intramembrane proteolysis is fundamentally accomplished. Given the tools that have been developed recently, this line of investigation promises to result in major advances in the coming years.

In contrast, deciphering the biological roles played by rhomboid enzymes in different organisms has lagged behind. Recent progress has both advanced our knowledge, and highlighted remarkable gaps in our understanding. In addition to conventional genetic approaches, deciphering the biochemistry of these enzymes is also likely to translate into new biological insights. To date, predicting substrates has been the only aspect of rhomboid biochemistry that has been useful in illuminating biology (Urban and Freeman 2003). However, this is likely to change, especially since linking rhomboid function to parasite invasion provides a strong incentive to studying these enzymes further, including developing inhibitors. In addition to being important therapeutics, such compounds would be ideally suited for a chemical biology approach to studying such a widespread family of enzymes, and this would reveal rhomboid function in a broad range of organisms in parallel. As we delve deeper into understanding its fundamental biophysics, as well as the commonality and differences of its biological roles, these advances may shed light on why this

type of biochemical mechanism has been apparently so useful at a relatively early point in the evolution of complex biological systems.

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CHAPTER 5

PROTEASES OF THE RHOMBOID FAMILY
IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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Abstract: Rhomboid proteins are a class of serine proteases conserved in all kingdoms of organisms. They contain six or seven transmembrane helices and control a wide range of cellular functions and developmental processes by intramembrane proteolysis. In yeast, two members of the rhomboid family are known, Rbd2 and Pcp1. Rbd2 is associated with the Golgi apparatus, but its function and its substrates are still unknown. The rhomboid protease Pcp1, located in the mitochondrial inner membrane, catalyses the second step in the proteolytic processing of cytochrome *c* peroxidase, a mitochondrial enzyme that acts as a peroxide scavenger. Pcp1 also affects the morphology of mitochondria by acting on, Mgm1, a dynamin-related GTPase. Mgm1 is present in short and long forms, and both isoforms are required for fusion of mitochondria and the maintenance of mitochondrial DNA. The proteolytic conversion of the long to the short form is catalysed by Pcp1. The cleavage sites in their substrates are not typical transmembrane domains but hydrophobic regions that must be actively translocated into the inner mitochondrial membrane by an ATP-consuming process to make them accessible to cleavage by the rhomboid protease

1.   INTRODUCTION

Many cellular functions are regulated by proteolysis. One recently discovered class of proteases, the so-called rhomboid proteases, catalyses the cleavage of membrane proteins within their membrane-spanning domains. These enzymes are themselves integral membrane proteins belonging to a subfamily of serine proteases, the first of which was identified by analysing the *Drosophila* developmental mutant for which they were named. In *Drosophila*, Rhomboid 1 processes the membrane-bound precursor of the epidermal growth factor (EGF), referred to as Spitz, into its active soluble form. This soluble ligand can then activate the EGF receptor,

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which belongs to a family of tyrosine kinases essential for growth and development (Urban *et al.* 2002). Rhomboid proteins have been conserved during evolution and have been found in organisms ranging from Archaea through Bacteria to lower eukaryotes like yeast, to plants and Metazoa. As bacteria and yeast have no EGF receptors, rhomboid proteases must serve functions other than processing of hormone precursors. The yeast *Saccharomyces cerevisiae* contains two genes for members of the rhomboid family: (i) *PCP1 / YGR101w* (Esser *et al.* 2002; this gene was named *RBD1* by McQuibban *et al.* 2003) and (ii) *RBD2/ YPL246c* (McQuibban *et al.* 2003).

2.   THE *RBD2* GENE OF YEAST ENCODES A RHOMBOID PROTEIN LOCATED IN THE SECRETORY PATHWAY

The two rhomboid genes of yeast, *RBD2* and *PCP1*, show low sequence homology to each other and to the rhomboid genes of other organisms. The Rbd2 protein is located in the secretory pathway and colocalises with the Golgi apparatus. Eight putative interactors for Rbd2p have been identified by yeast two-hybrid analyses (Drees *et al.* 2001; Tong *et al.* 2002; Uetz *et al.* 2000). These proteins are involved either in Golgi vesicle transport or actin polymerisation. However, nothing is yet known about the function of RBD2, as its actual substrates remain to be defined. Deletion of the gene results in no obvious phenotype (Giaever *et al.* 2002).

3.   THE RHOMBOID PROTEIN PCP1 IS FOUND IN YEAST MITOCHONDRIA

The second rhomboid gene, *PCP1*, encodes a protease located within the inner mitochondrial membrane. Deletion of the *PCP1* gene results in slow growth on non-fermentable carbon sources (Esser *et al.* 2002) and in fragmentation of mitochondria (Herlan *et al.* 2003; McQuibban *et al.* 2003; Sesaki *et al.* 2003). In addition, a slight shortening of telomere lengths (> 50 bp shorter than wild type) has been described in the deletion mutant (Askree *et al.* 2004). The significance of this telomere shortening, if any, is unknown.

Two substrates have been identified for the mitochondrial rhomboid protease so far: cytochrome *c* peroxidase (Ccp1p), an enzyme that acts as a scavenger of peroxides and toxic radicals, and Mgm1, a protein of the dynamin family that is required for mitochondrial fusion.

3.1.   The Mitochondrial Rhomboid Protease Pcp1 Catalyses the Proteolytic Processing of Cytochrome *c* Peroxidase

Cytochrome *c* peroxidase (Ccp1p) of yeast is synthesised with a bipartite 68-amino acid, N-terminal mitochondrial targeting sequence, which is removed in two steps to release the mature protein into the intermembrane space (IMS). Analysis of yeast deletion mutants identified three genes involved in the maturation of the cytochrome
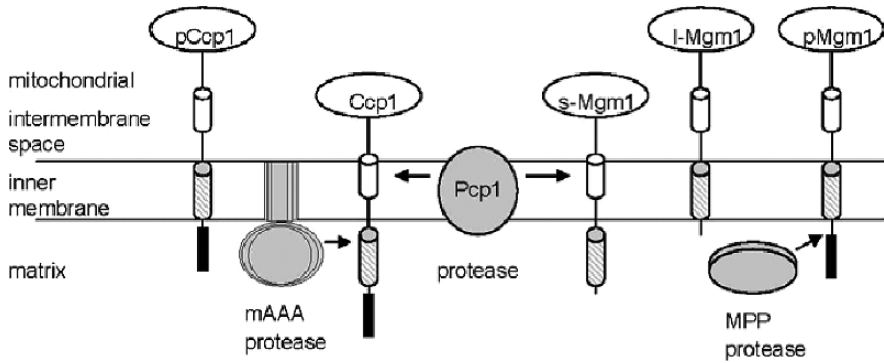
Figure 1. Model illustrating proteolytic processing of the two substrates of the mitochondrial rhomboid protease Pcp1. The matrix targeting signal sequences (thick lines) direct the precursors of Ccp1 and Mgm1 into mitochondria and the hydrophobic transmembrane domains (filled bars) anchor the proteins in the inner membrane. The signal sequence of Mgm1 is removed by the matrix processing peptidase MPP, resulting in the long form of Mgm1 (l-Mgm1) which remains bound to the inner membrane. In the presence of ATP, the import motor and/or the mAAA protease, the transmembrane domains are further translocated, permitting a second hydrophobic segment (open bars) to enter the inner membrane. The mAAA protease removes the transmembrane domain of the Ccp1 precursor, while the proteolytic activity of Pcp1 releases the mature Ccp1 and the short form of Mgm1 (s-Mgm1) into the intermembrane space

c peroxidase (Esser *et al.* 2002). Deletion of *YTA10* or *YTA12*, which together code for the two subunits of the mAAA protease, causes accumulation of the cytochrome c peroxidase precursor. This indicates that the first processing step is catalysed by the mAAA protease and demonstrates that the mAAA protease, which was previously shown to be involved in the degradation of misfolded and truncated proteins (Langer 2000), acts as a signal peptidase as well. As the catalytic site of mAAA is located on the matrix side of the inner membrane (Arlt *et al.* 1996), the N-terminal segment of the cytochrome c peroxidase precursor must first be translocated across the IM probably involving a pulling mechanism by the mAAA protease (Kihara *et al.* 1999; Leonhard *et al.* 2000). This not only allows cleavage by the mAAA, but also causes a second hydrophobic region of the precursor to enter the membrane and interact with the rhomboid peptidase (Fig. 1; Michaelis *et al.* 2005). After removal of the second part of the presequence by Pcp1p, the mature cytochrome c peroxidase is released into the mitochondrial intermembrane space to fulfil its function. Pcp1 was named for this activity (processing of cytochrome c peroxidase).

3.2. The Proteolytic Activity of Pcp1 Affects the Morphology of Mitochondria

Yeast mitochondria are dynamic organelles that are subject to fusion and fission. When fusion is blocked, continuing fission leads to extensive fragmentation of mitochondria with associated loss of mitochondrial DNA. Mgm1, a dynamin-related

GTPase, is required for mitochondrial fusion, and yeast strains in which the *MGM1* gene has been deleted accumulate fragmented mitochondria and lose mitochondrial DNA (Herlan *et al.* 2003; Sesaki *et al.* 2003b; Wong *et al.* 2003). Both the fragmentation of mitochondria and the loss of mitochondrial DNA in Δ*mgm1* cells can be prevented if mitochondrial division is prevented by inactivation of the Dnm1 protein, another dynamin-related GTPase.

The Mgm1 protein exists in two isoforms, a long (l-Mgm1) and a short (s-Mgm1) form. The long form contains an N-terminal transmembrane segment that is absent from the short form.

The peptidase that generates the short isoform was identified by different methods. Sesaki *et al.* (2003a) identified the rhomboid peptidase gene *PCP1* as *UGO2* in a genetic screen for components involved in mitochondrial fusion. Mutants were selected that maintained mtDNA in the absence of Dnm1p activity but lost it in the presence of Dnm1p. These mutants were transformed with a yeast genomic library and the transformants were screened for the ability to retain mtDNA in the presence of functional Dnm1p.

Herlan *et al.* (2003) screened cell extracts from yeast mutants deficient in mitochondrial proteases. The long form of Mgm1 which was generated by the matrix processing peptidase MPP undergoes further proteolytic cleavage by the rhomboid protease Pcp1 (Fig. 1). The short form of Mgm1 is missing in a *pcp1* deletion mutant. These authors found that both the long and the short isoforms of Mgm1 are essential for the maintenance of normal mitochondrial morphology and mitochondrial DNA, and both forms are required to complement the phenotype of an *mgm1* deletion.

McQuibban *et al.* (2003) selected potential substrates of Pcp1 from yeast genome databases based on two criteria: mitochondrial localisation and the presence of a single predicted transmembrane domain which is missing in the processed proteins. The two substrates Ccp1 und Mgm1 fulfil these criteria.

Cells with in which *PCP1* is missing contain partially fragmented mitochondria and few tube-shaped organelles (Herlan *et al.* 2003; McQuibban *et al.* 2003; Sesaki *et al.* 2003a). This fragmentation is associated with a marked loss of mitochondrial DNA. Thus the mitochondrial phenotypes associated with *PCP1* deletion seem to be due to the defect in processing of Mgm1, since lack of processing of cytochrome c peroxidase, the second substrate of the Pcp1 protease, causes only peroxide sensitivity (our unpublished results).

However, in contrast to the *mgm1* deletion mutant – in which mitochondrial fusion is inhibited – the *pcp1* deletion strain remains competent for mitochondrial fusion (Sesaki *et al.* 2003a). This result in turn is inconsistent with the assumption that both forms of Mgm1 are required for mitochondrial fusion (Herlan *et al.* 2003). Does it indicate that, in yeast, only the long form of Mgm1 is required for mitochondrial fusion and that the short form has another function? Further experiments will be required to clarify this question, but such a possibility is suggested by studies on Opa 1, the human ortholog of Mgm1 (Cipolat *et al.* 2006; Ishihara *et al.* 2006).

Mutations in the human *OPA1* gene cause an autosomal dominant disease, optic atrophy type 1, a progressive degeneration of the optic nerve leading to blindness (Delettre *et al.* 2002).

Eight Opa1 splice variants have been described which are processed to various isoforms (Delettre *et al.* 2001). Two variants were analysed for processing and mitochondrial morphology by Ishihara *et al.* (2006). According to these authors, only the long membrane-bound isoform stimulates mitochondrial fusion, whereas the short isoform stimulates mitochondrial fragmentation and apoptosis. Cipolat *et al.* (2006) agree that only the long forms of Opa1 are required for mitochondrial fusion; however, they conclude that the processed forms of Opa1 have anti-apoptotic activity.

Several reports indicate that Parl, the human homologue of Pcp1, plays a role in Opa1 processing. A protease function for Parl was first suggested by complementation analysis of the yeast *pcp1* mutant (McQuibban *et al.* 2003). Expression of *PARL* in a Δ*pcp1* yeast strain restores processing of Ccp1 and Mgm1, growth on non-fermentable carbon sources and mitochondrial morphology, indicating that *PARL* can functionally substitute for *PCP1*.

A direct interaction between Parl and Opa1 was demonstrated by co-immunoprecipitation of both proteins with anti-Parl antibody (Cipolat *et al.* 2006). The increased levels of soluble Opa1 protein in the intermembrane space fraction seen in wild-type mitochondria relative to Parl$^{-/-}$ mitochondria suggests that Opa1 is indeed a substrate of Parl. The existence of at least one additional protease that cleaves Opa1 is postulated because some Opa1 protein remains detectable in the IMS fraction of Parl$^{-/-}$ mitochondria.

It should be mentioned here that Ishihara *et al.* (2006) were unable to detect any effect on Opa1 processing after repression of the endogenous or expression of an introduced exogenous *PARL* gene in transfected HeLa cells.

Interestingly, loss of Rhomboid 7, the mitochondrial rhomboid of *Drosophila*, induces apoptosis, but Urban *et al.* (2006) have suggested that apoptosis may not be a primary phenotype. Defects in mitochondrial fusion during fly spermatogenesis and muscle maturation may cause the phenotype of *rho7* mutants. These mutant flies show neurological defects, are unable to fly and have difficulty walking. They live for only three days, whereas wild-type flies have a typical lifespan of about 60 days.

3.3.   Proteolytic Activity of Pcp1

Rhomboid 1 (Rho1) of *Drosophila* was first classified as a serine protease with a catalytic triad by studying the effects of replacing conserved amino acids (Urban *et al.* 2001). The catalytic triad (S-H-N) resembles that of the classical soluble serine proteases (S-H-D), except that the aspartate residue is replaced by an asparagine. Each of these catalytic residues resides in a different transmembrane domain. Intramembrane proteolysis activity has been reconstituted using purified rhomboid proteins from various organisms (Lemberg *et al.* 2005; Urban and Wolfe 2005).

These *in vitro* assays revealed that neither ATP nor any cofactors are required for proteolysis. Recent analysis of purified Rhomboid 1 proteins from mutants, however, suggests that rhomboids contain a catalytic dyad rather than the proposed triad (Lemberg *et al.* 2005). Whereas serine and histidine participate in catalysis, asparagine seems not to be required. This proposal is supported by the first three-dimensional structure to be reported for a rhomboid protease by Wang *et al.* (2006).

The yeast rhomboid protease Pcp1 is a serine protease like the other members of the rhomboid family. The yeast enzyme contains a serine protease motif (GASGA) similar to that found in Rho1 of *Drosophila* (GASGG) (Fig. 2). When the serine residue is replaced by isoleucine (S256I) the proteolytic activity of the yeast protein is abolished and the intermediate form of cytochrome *c* peroxidase accumulates (Esser *et al.* 2002). The serine protease motifs of rhomboid proteins known to be located in mitochondria are highly conserved. The yeast (ScPcp1), human (HsParl) and *Drosophila* (DmRho7) enzymes all contain the sequence SLGASGA (Fig. 2). The conserved S252 and G254 residues do not participate in catalysis: replacement of either by alanine does not eliminate the protease activity of the yeast enzyme (Esser *et al.* 2002, and our unpublished results). Thus, the serine S256 is most likely to be the catalytic centre of the yeast enzyme.

The histidine H313 of the yeast Pcp1 protease is required for proteolytic activity, as in the *Drosophila* Rho1 (DmRho1). In addition, mitochondrial DNA is lost in mutant cells in which histidine H313 is replaced by alanine (Sesaki *et al.* 2003). This mutation prevents proteolytic processing of Ccp1 to the mature protein (our

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                      *  ***     *  **      **
ScPcp1   184   ISIIGSAFSHQEFWHLGMNMLALWSFGT
HsParl   207   SPMLLSTFSHFSLFHMAANMYVLWSFSS
DmRho7   186   WPMFLSTFSHYSAMHLFANMYVMHSFAN
DmRho1   151   WRFFSYMFLHANWFHLGFNIVIQLFFGI
                  *  *      *     *         *


               *  *******     *
ScPcp1   250   GPSLGASGALFGVLGCFSYLF
HsParl   271   GPSLGASGAIMTVLAAVCTKI
DmRho7   250   GMSLGASGAIMTLLAYVCTQY
DmRho1   211   VFLVGASGGVYALLAAHLANI
                  ****        *


               ** *******    *        *   *
ScPcp1   308   FDYAAHLGGSMMGVLYGWYISKAVEKQ
HsParl   330   FDHAAHLGGALFGIWYVTYGHELIWKN
DmRho7   309   FDHAAHLGGAMFGIFWATYGAQIWAKR
DmRho1   276   VSYIAHLTGALAGLTIGFLVLKNFGHR
                  *** *      *
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Figure 2. Alignment of the mitochondrial rhomboid proteases of yeast (ScPcp1), human (HsParl) and *Drosophila* (DmRho7) and Rho1 of *Drosophila* (DmRho1). The putative catalytic triad composed of three transmembrane domains is shown. Stars above: residues identical in all three mitochondrial rhomboid proteases. Stars below: residues identical in the four rhomboid proteases shown. WR: residues conserved in all non-mitochondrial rhomboid proteases of *Drosophila melanogaster*

unpublished results). The residues adjacent to histidine H313 are conserved in yeast, human and *Drosophila*, especially in the mitochondrial members of the rhomboid family (Fig. 2).

The region adjacent to the asparagine residue N202, proposed to be part of the catalytic triad, is much less conserved (Fig. 2). However, Sesaki *et al.* (2003a) reported a respiratory deficiency in the N202A yeast mutant (called N212A by these authors), suggesting that this asparagine residue is required for the *in vivo* function of the yeast enzyme. Further experiments have to show whether the yeast enzyme contains a catalytic triad or dyad (see above).

3.4.   Substrate Specificity

Rhomboid 1 of *Drosophila* recognises a small region in the transmembrane domain of its substrate Spitz and this region is sufficient for cleavage (Urban and Freeman 2003). It seems that a specific sequence is not recognized but helix-breaking residues are crucial.

The sites cleaved by Pcp1 in its two substrates are known. Pcp1 cleaves Mgm1 between residues 160 and 161, as determined by sequence analysis of the N-terminus of s-Mgm1 (Herlan *et al.* 2003). Sixty-eight amino acid residues are removed (in two steps; see above) from the cytochrome *c* peroxidase precursor (Kaput *et al.* 1982). Pcp1 cleaves both substrates downstream of a threonine residue (Fig. 3). This ß-branched residue is thought to destabilise helical structures, as do proline and glycine residues (Chou and Fasman 1974). The region of the cleavage site in l-Mgm1 is rich in glycine residues, whereas helix-destabilizing residues are less prominent in the cytochrome *c* peroxidase sequence. Nevertheless, the substrate specificity of Pcp1 seems to be similar to that of the cytosolic rhomboids of *Drosophila*.

Interestingly, Pcp1 recognises hydrophobic sequences that are not predicted to be transmembrane domains. These hydrophobic sequences are located downstream of transmembrane domains which have to be translocated into the mitochondrial matrix by the import motor and/or the mAAA protease in an ATP-consuming process before the hydrophobic sequences enter the inner membrane and become accessible
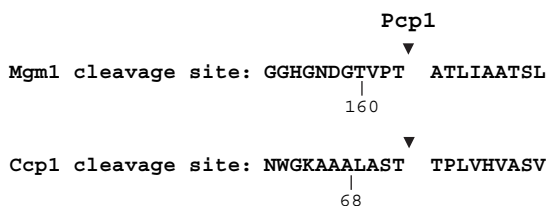
```
                              Pcp1
                               ▼
Mgm1 cleavage site: GGHGNDGTVPT  ATLIAATSL
                               |
                              160

                               ▼
Ccp1 cleavage site: NWGKAAALAST  TPLVHVASV
                               |
                              68
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Figure 3. Cleavage sites used by the mitochondrial rhomboid protease Pcp1 of yeast. The substrate cytochrome c peroxidase (Ccp1) is cleaved between residues 68 and 69 (Kaput *et al.* 1982) and the dynamin-related protein Mgm1 between residues 160 and 161 (Herlan *et al.* 2003)

to the rhomboid protease Pcp1 (Fig. 1; Esser *et al.* 2002; Herlan *et al.* 2004; Michaelis *et al.* 2005).

4.   OUTLOOK

Rhomboid proteins are found in all kingdoms of life, suggesting that they arose early in evolution. Nevertheless the functions of most of these conserved membrane proteins remain unknown. One example is the yeast rhomboid protein Rbd2 associated with the secretory pathway. Its proteolytic activity and substrates remain to be defined.

The second rhomboid protein of yeast, Pcp1, influences the morphology of mitochondria, but how proteolytic cleavage of Mgm1 affects mitochondrial fusion needs further analyses. The function of the short form of Mgm1 is controversial and needs to be reinvestigated.

There are a number of additional open questions concerning the mitochondrial rhomboid protease of yeast. For example:
– how is the rhomboid protease itself imported and processed?
– does the proteolytic activity of Pcp1 require any cofactor or ATP?
– does Pcp1 interact with other proteins of the inner mitochondrial membrane, for example with the mAAA protease required for maturation of cytochrome *c* peroxidase?
– does Pcp1 influence the replication and distribution of mitochondrial DNA?
– are cytochrome *c* peroxidase and Mgm1 the only substrates of Pcp1?

The analysis of mitochondrial rhomboid proteases of yeast and other organisms may also help us to understand how the human rhomboid proteases influence pathological conditions like diabetes and Alzheimer's disease, in which intramembrane proteolysis is thought to play a role.

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CHAPTER 6

γ-SECRETASE AND ALZHEIMER'S DISEASE

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Abstract: Deposition of the amyloid β-protein is a defining pathological characteristic of Alzheimer's disease, and this small protein is proteolytically produced from the amyloid β-protein precursor. γ-Secretase is responsible for the second cut, which forms the C-terminus of amyloid-β and determines how much of the transmembrane domain is included in this aggregation-prone protein. This intramembrane aspartyl protease is a complex of four different integral membrane proteins: presenilin, nicastrin, Aph-1 and Pen-2. During assembly and maturation of the protease complex, presenilin is endoproteolyzed into two subunits, each of which contributes one aspartate to the active site. A model of successive proteolysis may explain how Alzheimer-causing mutations in presenilin can both decrease enzyme activity and increase the proportion of longer, more aggregation-prone forms of amyloid-β. Substrate apparently interacts with an initial docking site before passing in whole or in part between the two presenilin subunits to the internal water-containing active site. The ectodomain of nicastrin also interacts with the N-terminus of the substrate as an essential step in substrate recognition and processing. Inhibitors and allosteric modulators of γ-secretase activity are under investigation as potential Alzheimer therapeutics. Elucidation of detailed structural features of γ-secretase is the next logical step toward understanding how this enzyme carries out intramembrane proteolysis and will set the stage for structure-based drug design

Keywords: amyloid β-protein, amyloid β-protein precursor, presenilin, inhibitors

1. INTRODUCTION

Within the cerebral cortex and limbic system of the Alzheimer brain are found deposits, or plaques, primarily composed of the 4 kDa amyloid-β peptide (Aβ) (Glenner and Wong, 1984). This peptide is clipped out of a 110-120 kDa type I integral membrane protein called the Aβ protein precursor (APP) by the sequential action of two proteases, β-and γ-secretases (Fig. 1) (Selkoe, 2001). The discovery of genetic mutations in the APP genes that cause early-onset hereditary Alzheimer's disease strongly suggested that Aβ was a key pathogenic player: these mutations
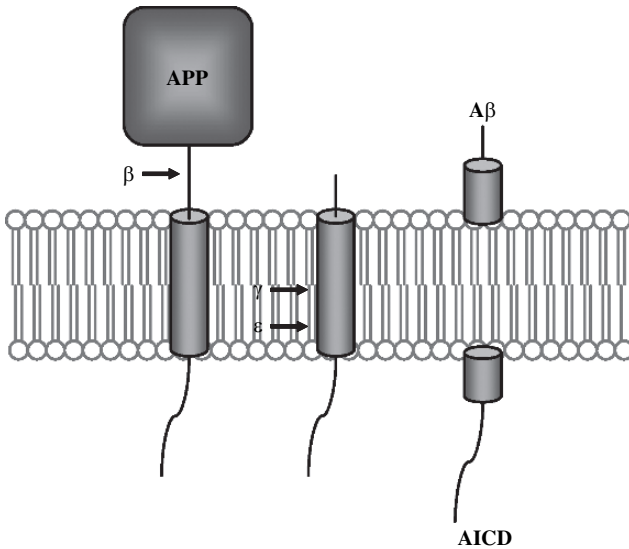
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Figure 1. Proteolytic processing of APP. The ectodomain of APP is first shed by β-secretase. Alternatively, APP can be cut within the Aβ region by α-secretases (not shown). The remaining membrane-associated stub is cleaved at least twice in the transmembrane region, at the γ site to produce Aβ and at the ε site to produce the intracellular domain (AICD). The transmembrane cleavage events are carried out by the presenilin-containing γ-secretase complex

were found within the region encoded by Aβ or immediately adjacent to β- and γ-secretase cleavage sites (Hardy, 1997). Indeed, these mutations alter either the properties of Aβ or how much and what type of Aβ is produced. Those mutations near the N-terminus of the Aβ region of APP increase cleavage by β-secretase, resulting in increased Aβ (Cai *et al.*, 1993; Citron *et al.*, 1992). Those near the γ-secretase site, however, have a more subtle effect, changing the proportion of 40- to 42-residue forms of Aβ (Suzuki *et al.*, 1994). Although the latter is a minor Aβ variant, it is highly prone to aggregation and represents the majority of Aβ found in Alzheimer plaques (Iwatsubo *et al.*, 1994; Roher *et al.*, 1993).

Another major clue to Alzheimer pathogenesis came with the discovery of two related genes, presenilin-1 (PS1) and presenilin-2 (PS2), likewise associated with early-onset disease (Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995; Sherrington *et al.*, 1995). The presenilin genes encode ∼50 kDa multi-pass membrane proteins. At the time, the only gene with even distant homology to the presenilins was found in worms and only known to play a role in spermatogenesis (L'Hernault and Arduengo, 1992). How this was related to Alzheimer's disease could not have been more obscure. Nevertheless, it became clear that the Alzheimer mutations in the presenilins alter Aβ production (Citron *et al.*, 1997; Duff *et al.*, 1996; Lemere *et al.*, 1996; Scheuner *et al.*, 1996). Over 100 such missense mutations have been identified so far, and the vast majority of those examined in detail

skew the proportion of Aβ toward the more aggregation-prone 42-residue form (Tanzi and Bertram, 2005). Thus, the presenilin mutations change the cleavage site specificity of γ-secretase. The subsequent discovery that knockout of presenilin-1 dramatically reduced γ-secretase cleavage of APP suggested that presenilin mediates this transmembrane proteolytic event (De Strooper et al., 1998).

On the heels of the discovery of presenilins as Alzheimer genes came the identification of a close homolog in *C. elegans* that facilitates Notch signaling (Levitan and Greenwald, 1995), and it soon became clear that proteolysis of Notch was essential for signaling from this receptor (see next chapter by R. Kopan). Upon contact with its cognate ligand, the Notch receptor undergoes ectodomain shedding followed by proteolysis of the membrane-associated stub within its transmembrane region (Schroeter et al., 1998). The released intracellular domain then translocates to the nucleus, interacting with certain transcription factors and coactivators to regulate gene expression critical for determining cell fate. Intriguingly, knockout of presenilin-1 in mice resulted not in neurodegeneration but in embryonic lethality and a phenotype remarkably similar to that seen upon knockout of Notch1 (Shen et al., 1997; Wong et al., 1997). Culturing of cells from these knockout embryos revealed that presenilin is not only needed for the γ-secretase cleavage of APP, but also for proteolysis of the Notch transmembrane domain (De Strooper et al., 1999).

2.   PRESENILIN AS THE CATALYTIC COMPONENT OF γ-SECRETASE

Membrane topology experiments with presenilin gave some conflicting results, with suggestions of six, seven, or eight transmembrane domains, and with the N-terminus being either lumenal/extracellular or cytosolic (Kim and Schekman, 2004). However, the strongest evidence originally favored eight transmembranes, with the N-terminus, large cytosolic loop, and C-terminus all on the cytosolic side (Doan et al., 1996; Li and Greenwald, 1996, 1998). Most recently, this topology has been updated and refined, to nine-transmembrane domains (Laudon et al., 2005; Oh and Turner, 2005), with the C-terminus on the lumenal/extracellular side but folded into the protein or its partners (see below). A biochemical clue to the function of presenilin came with the observation that the protein is endoproteolyzed into two pieces (Fig. 2), an N-terminal fragment (NTF) and a C-terminal fragment (CTF) that remain associated, have a long biological half life together, and are tightly regulated by limiting cellular factors (Ratovitski et al., 1997; Thinakaran et al., 1996; Thinakaran et al., 1997). These findings suggested that the NTF/CTF heterodimer is the mature, active form of presenilin, whatever its biochemical function might be. Meanwhile, the design of substrate-based peptidomimetic inhibitors for γ-secretase suggested that the enzyme is an aspartyl protease: hydroxyl-containing transition-state mimics could block APP processing at the γ-secretase level in cells (Shearman et al., 2000; Wolfe et al., 1999b).

Given evidence that γ-secretase is an aspartyl protease and that presenilin is critical for γ-secretase activity, presenilin was examined for the presence of two
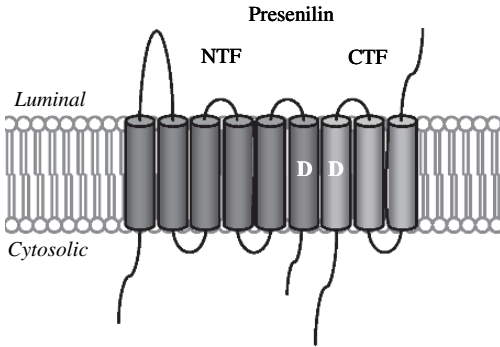
Figure 2. Membrane topology of presenilin, a putative aspartyl protease. Presenilin undergoes endopro-
teolysis into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) that remain associated.
Two conserved aspartates within adjacent transmembrane domains are essential for both presenilin
endoproteolysis and γ-secretase activity

aspartates that might be part of a novel protease. Two completely conserved trans-
membrane aspartates, one in the NTF domain and one in the CTF domain (Fig. 2),
were identified and found to be independently essential for both presenilin endopro-
teolysis and γ-secretase cleavage of APP (Wolfe *et al.*, 1999a; Wolfe *et al.*, 1999c).
This discovery suggested that presenilin was indeed a novel aspartyl protease, and
one activated by autoproteolysis. Upon maturation into NTF and CTF, the active
site of γ-secretase was proposed to lie at the interface between these two subunits,
each of which contributed one of the catalytic aspartates. Further validation for
this hypothesis came from affinity reagents designed from transition-state analogue
γ-secretase inhibitors, which directly bound to presenilin NTF and CTF (Esler
et al., 2000; Li *et al.*, 2000). These affinity labeling reagents, directed to the
γ-secretase active site, tagged heterodimeric presenilin, identifying this interface as
the protease catalytic site.

Presenilin alone, however, was clearly not the whole of γ-secretase. Consistent
with the need for limiting cellular factors to gate presenilin endoproteolysis, the
protein and its fragments were found to enter into high molecular weight complexes
(Capell *et al.*, 1998; Yu *et al.*, 1998). Moreover, presenilin alone did not cleave
itself, and a mutant presenilin that does not require endoproteolysis to support
γ-secretase activity in cells did not display proteolytic activity on its own either (i.e.
limiting cellular factors were necessary). However, key support for presenilin as
a protease came with the discovery of signal peptide peptidase (SPP) (see chapter
by T. Golde and A. Nyborg). This enzyme, which clips remnant signal peptides in
the membrane after their release by signal peptidase, was discovered using affinity
labeling with a transition-state analogue inhibitor, and the responsible protein bore
clear similarity to presenilin, including two highly conserved transmembrane motifs,
each containing an aspartate (Weihofen *et al.*, 2002). Unlike presenilin, SPP appar-
ently supported proteolytic activity on its own, without the need for limiting cellular
factors or endoproteolysis. Thus, if a presenilin-like protein such as SPP is a

protease, presenilin itself is most likely the catalytic component of γ-secretase. Nevertheless, it is clear that presenilin has important non-proteolytic functions as well, independent of its role in γ-secretase activity [e.g., (Baki *et al.*, 2004; Huppert *et al.*, 2005; Kang *et al.*, 2002)]. These other functions, however, lie outside the scope of this chapter.

3.   HOW PRESENILIN MUTATIONS CAUSE FAMILIAL ALZHEIMER'S DISEASE

In parallel with the discovery of presenilin as a protease that cleaves APP and Notch, Alzheimer-causing mutations in presenilin were found to possess reduced proteolytic function. Yankner and colleagues first showed this effect with a variety of mutant presenilins using a Notch-based luciferase reporter assay (Song *et al.*, 1999). Several other groups have since noted this phenomenon (Lewis *et al.*, 2000), the most recent from De Strooper and colleagues, who showed that mutations in presenilin reduced its proteolytic function towards several different substrates (Bentahir *et al.*, 2006). These findings raise an apparent paradox, in which Alzheimer-causing disease mutations cause both a "gain of function" (an increase in Aβ42/Aβ40) and a "loss of function" (a decrease in proteolytic activity). These seemingly opposing effects have elicited considerable debate over how the presenilin mutations cause Alzheimer's disease, with some even suggesting that reducing Aβ production with candidate therapeutics might actually exacerbate or cause the disease.

To appreciate the resolution of this purported paradox, it should first be noted that the presenilin-containing γ-secretase complex cuts the transmembrane domain of APP (and other substrates) in at least two positions: the γ site that produces the carboxy-terminus of Aβ and the ε site further downstream that produces the amino-terminus of the APP intracellular domain (AICD; Fig. 1) (Weidemann *et al.*, 2002). Whereas cleavage at the γ site is heterogeneous—producing Aβ peptides of 39–43 residues—the cut at the ε site produces almost exclusively a 50-residue AICD. The same phenomenon occurs with Notch: heterogenous cleavage in the middle of the transmembrane domain (the S4 site) and homogeneous cleavage further downstream (at the S3 site (Okochi *et al.*, 2002). Interestingly, proteolysis at these two sites is affected by Alzheimer-causing mutations in APP and the presenilins, which lead to an increase in the proportion of Aβ42 relative to Aβ40 along with an increase in a new 51-residue AICD relative to the 50-residue product (Sato *et al.*, 2003). Thus, these two proteolytic events are not completely independent: a change in the cleavage site in one correlates with a change in the cleavage site of the other. [However, it should be pointed out that, in one study, several artificial mutations of L166 in PS1 increased Aβ42 production without affecting AICD levels (Moehlmann *et al.*, 2002), and in another report, inhibition of endocytosis altered AICD formation without changing Aβ42/Aβ40 (Fukumori *et al.*, 2006).]

Recent evidence from Ihara and colleagues suggests that the ε-cleavage event takes place before proteolysis at the γ site. First, analysis of intracellular Aβ reveals a very small but significant amount of longer forms of this peptide, up to Aβ49, which

is the proteolytic counterpart to the 50-residue AICD (Qi-Takahara *et al.*, 2005). By contrast, longer AICDs (for example, AICD counterparts to Aβ40 or Aβ42) have not been detected. Second, expression of Aβ49 leads to the secretion of Aβ40 and Aβ42 in the same proportion that is produced by γ-secretase (Funamoto *et al.*, 2004). Third, swapping tryptophan residues into the γ site within the APP transmembrane domain prevents γ cleavage but allows ε cleavage; however, swapping tryptophans into the ε site leads to proteolysis in between the γ and ε sites, at a so-called ζ site (Fig. 1 and see below)(Sato *et al.*, 2005). Installing tryptophans into the ζ site prevents any transmembrane cleavage of APP. Thus, with these tryptophan swaps, ε cleavage can occur without γ cleavage, but γ cleavage is not seen without ε or ζ cleavage. (Interestingly, longer Aβ peptides resulting from cleavage at the ζ site are seen intracellularly on treatment with one particular γ-secretase inhibitor, a dipeptide analogue called DAPT (Yagishita *et al.*, 2006)). Finally, a mutation in PS1 (M233T) leads to alternative ε cleavage, producing the 51-residue AICD and its counterpart Aβ48 in a cell-free assay with detergent-solubilized membranes (Kakuda *et al.*, 2006).

One way to explain two major cleavage sites would be the presence of two pairs of catalytic aspartates within a presenilin dimer at the core of the γ-secretase complex. Several laboratories have reported evidence for a presenilin-presenilin interaction (for example Schroeter *et al.*, 2003). However, recent evidence from our laboratory suggests that immunoprecipitation of one epitope-tagged presenilin does not bring down a coexpressed, differentially tagged counterpart, but γ-secretase activity is nevertheless found in the immunoprecipitate (T. Sato, M. S. W., unpublished data). Thus, two presenilin molecules per complex are not required for proteolytic activity: one presenilin suffices to generate the normal γ-cleaved Aβ and ε-cleaved AICD. Moreover, cysteine mutagenesis and disulfide crosslinking experiments show that the key aspartate in transmembrane domain 6 is adjacent to the key aspartate in transmembrane domain 7, with no evidence for dimeric presenilin (Tolia *et al.*, 2006). Together, these findings support a model of the γ-secretase complex in which one presenilin (and thus one pair of aspartates) is sufficient to cut the transmembrane domain of APP and other substrates in at least two places.

Ihara and coworkers have suggested that these and other observations are consistent with successive cleavage events: initial proteolysis at the ε site leads to the release of AICD, but the long Aβ products (Aβ49 or Aβ48) remain in the active site and are successively cleaved every three residues upstream at the ζ sites and then again at the γ sites (Qi-Takahara *et al.*, 2005) Fig. 3). Specifically, they propose that Aβ49 is processed to Aβ46, Aβ43 and Aβ40, whereas Aβ48 is trimmed to Aβ45, Aβ42 and Aβ39. This model of successive proteolysis from the ε site to the γ sites elegantly explains how so many presenilin mutations can both reduce proteolytic activity (that is, cause a "loss of function") and increase the Aβ42/Aβ40 ratio (that is, cause a "gain of function"). Mutant versions of the enzyme that are less proteolytically efficient also cut proportionately more at the alternative ε site, producing Aβ48 (Sato *et al.*, 2003). The slower mutant enzymes allow proportionately more release of Aβ42 before further trimming to Aβ39. The
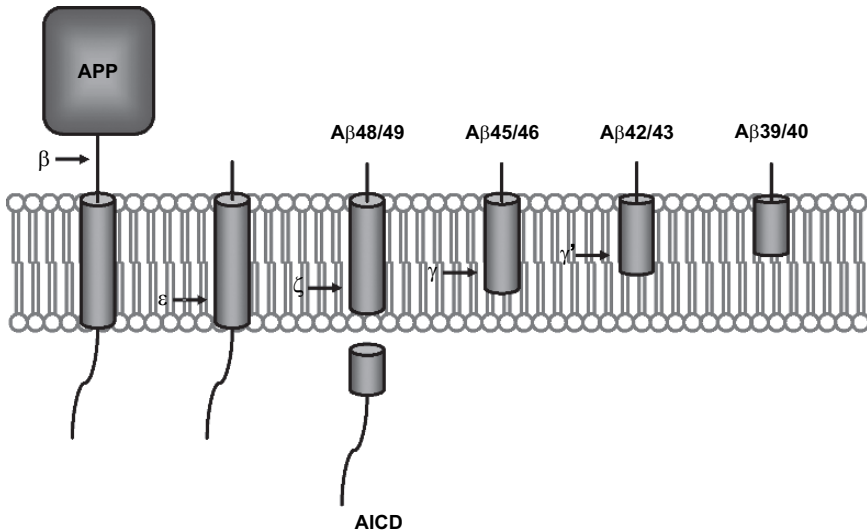
Figure 3. Ihara model of processive proteolysis of the APP transmembrane domain by γ-secretase, beginning at the ε cleavage site and cleaving every three residues. This model explains how reduction of proteolytic function owing to presenilin mutations might lower Aβ production but increase the ratio of Aβ42 to Aβ40. Longer forms of Aβ, with more of the hydrophobic transmembrane domain, might be more likely to be retained in the active site of the protease, whereas the shorter forms are more likely to be released. Less catalytically efficient γ-secretase complexes would allow more time for the release of longer Aβ peptides. In addition, Alzheimer disease-causing presenilin mutations shift the initial ε cleavage site to produce more Aβ48, which would lead to Aβ42. AICD, APP intracellular domain; APP, amyloid precursor protein

net result might be less total Aβ, including less Aβ40 (Bentahir *et al.*, 2006), but the ratio of Aβ42 to Aβ40 is elevated. In cases in which the enzyme's proteolytic efficiency is only slightly reduced, the resulting increase in substrate levels might ultimately lead to compensation and little change in total Aβ; nevertheless, the reduced efficiency would cause an increase in Aβ42/Aβ40. This same mechanism might also account for the changes in Aβ42/Aβ40 seen with Alzheimer-causing APP mutations that are located near the γ-secretase cleavage sites: in these cases, the mutant substrates might be processed less efficiently by the wild-type protease.

The scenario described above, which is supported by numerous reports, also provides an explanation for why the deletion of three out of four presenilin alleles in mice, with only one PS1 allele remaining, does not result in elevated Aβ42/Aβ40 [although see (Refolo *et al.*, 1999)]: the remaining γ-secretase complexes are wild type, have normal proteolytic activity, and trim ε-cleaved Aβ efficiently. This is also consistent with the fact that although more than 100 missense mutations in PS1 and PS2 have so far been associated with Alzheimer's disease, none are complete loss-of-function mutations in an allele (for example, complete deletion, loss of expression, mutation of one of the catalytic aspartates, or severe truncations).

Conditional-knockout of presenilins in the brain can apparently result in memory impairment and age-dependent neurodegeneration (Saura *et al.*, 2004), but without either Aβ-containing plaques or tau-containing tangles, this is arguably not a phenocopy of Alzheimer's disease. Apparently, presenilin is crucial for neuronal survival and/or replacement in the brain, and complete knockout of both alleles has serious consequences. However, many other genes that are unrelated to Alzheimer's disease but likewise needed for maintaining neuronal density would be expected to do the same on complete knockout in the adult brain.

4.   OTHER COMPONENTS OF THE γ-SECRETASE COMPLEX

Because expression of presenilin alone did not increase γ-secretase activity, presenilin required limiting cellular factors to mature into two subunits, and presenilin entered into high-molecular weight complexes, it was clear that γ-secretase was composed of more than just presenilin. The search was on to identify other members of what would become known as the γ-secretase complex. The first to be discovered was nicastrin, a presenilin-interacting protein found by co-isolation upon immunoaffinity purification (Yu *et al.*, 2000). Nicastrin is a highly glycosylated 120–140 kDa type I integral membrane protein, with almost the entire protein being lumenal/extracellular. RNAi knockdown experiments demonstrated that nicastrin was essential for the γ-secretase cleavage of both APP and Notch (Yu *et al.*, 2000). Nicastrin was also shown to be required for presenilin endoproteolysis, suggesting that it was at least one of the "limiting cellular factors" gating presenilin subunit formation (Yu *et al.*, 2000). Consistent with these observations, isolation of γ-secretase using an immobilized transition-state analogue inhibitor resulted in copurification of nicastrin, suggesting that nicastrin was indeed a bona fide member of the γ-secretase complex (Esler *et al.*, 2002). Nicastrin is found primarily as two bands by SDS-PAGE, the upper band being composed of a more highly glycosylated form that is especially associated with presenilin NTF and CTF subunits and active γ-secretase (Arawaka *et al.*, 2002; Edbauer *et al.*, 2002; Kimberly *et al.*, 2002; Leem *et al.*, 2002; Tomita *et al.*, 2002). As with presenilin, knockout of nicastrin in different species results in lethal phenotypes resembling those seen with Notch deficiencies (Goutte *et al.*, 2000; Li *et al.*, 2003).

However, overexpression of presenilin and nicastrin still did not result in increased presenilin endoproteolysis or γ-secretase activity, the implication being that other associated proteins were yet to be discovered. Genetic studies in *C. elegans* to identify new Notch modifiers revealed two novel genes, Aph-1 and Pen-2, which encode proteins of seven and two predicted transmembrane domains, respectively (Francis *et al.*, 2002; Goutte *et al.*, 2000). RNAi knockdown of these genes, as with nicastrin and presenilin, blocked γ-secretase cleavage of APP and Notch. Follow up studies demonstrated that overexpression of all four proteins together (presenilin, nicastrin, Aph-1, and Pen-2) resulted in increased levels of presenilin NTF and CTF, mature nicastrin, and γ-secretase activity (Kimberly *et al.*, 2003; Takasugi *et al.*, 2003). One study even showed this to be true in yeast

(Edbauer *et al.*, 2003), the genome of which does not encode for any homologs of these four proteins. Expression of all four proteins is required for these effects. In addition, coimmunoprecipitation of any one of the five proteins (PS1 NTF and CTF being separately examined) brought down all the others, indicating that they interact with each other. Partial purification of γ-secretase through several steps resulted in isolation of all five proteins (Kimberly *et al.*, 2003). Several groups have now reported purification to virtual homogeneity (Fraering *et al.*, 2004b; Shah *et al.*, 2005; Zhou *et al.*, 2005), providing definitive proof that these proteins form a single complex and are the essential components of γ-secretase. Purification has so far allowed low-resolution structural elucidation by electron microscopy (EM) coupled to single particle image analysis, suggesting that the complex contains a cylindrical 20–40 Å cavity and 20 Å pores at the top and bottom that might serve as entry ports for water (Lazarov *et al.*, 2006). The large internal cavity is reminiscent of the proteasome, and indeed, because of its role in clearing a variety of type I integral membrane stubs, γ-secretase has been dubbed the "proteasome of the membrane" (Kopan and Ilagan, 2004).

Despite full identification, purification and determination of a low-resolution structure, the stoichiometry of the protease complex remains unclear. Particularly vexing has been the issue of whether the complex contains two presenilin molecules at its catalytic core. Although some evidence supports this idea (Cervantes *et al.*, 2004; Hebert *et al.*, 2003; Schroeter *et al.*, 2003), including the finding that the presenilin homolog SPP forms an SDS-stable dimer (Nyborg *et al.*, 2004), confirmation from other laboratories has not been forthcoming, and even the EM structure cannot discern this clearly. Another complication is that six variants of the γ-secretase complex apparently exist, due to different combinations of the two presenilins and three different Aph-1 proteins (Shirotani *et al.*, 2004). Perhaps these different complexes have different affinities for the various γ-secretase substrates, which besides APP and Notch1 also include APP-like proteins APLP-1 and -2, Notch2-4, the Notch ligand Jagged, ErbB4, E- and N-cadherins, and the CD44 receptor (Kopan and Ilagan, 2004). Biochemical differences between these complexes no doubt exists; indeed, PS2-containing complexes display lower proteolytic activity than PS1-containing complexes (Lai *et al.*, 2003).

Assembly of the γ-secretase complex begins in the endoplasmic reticulum soon after translation and membrane insertion. Nicastrin and Aph-1 assemble into a subcomplex, with nicastrin remaining in an immature, hypoglycosylated form (Gu *et al.*, 2002). Presenilin and Pen-2 are added afterwards. Whether presenilin and Pen-2 interact with each other first before assembly with the nicastrin/Aph-1 subcomplex is unclear, but knockdown of Pen-2 does lead to a Nicastrin/Aph-1/Presenilin subcomplex in which presenilin remains as a holoprotein (LaVoie *et al.*, 2003; Luo *et al.*, 2003; Takasugi *et al.*, 2003). The addition of Pen-2 leads to presenilin NTF/CTF formation, maturation of nicastrin, and active γ-secretase. Partial dissociation of the γ-secretase complex using the nonionic detergent dodecyl-β-D-maltoside (DDM) followed by 2D PAGE analysis revealed how the γ-secretase components are arranged in the active protease complex (Fraering *et al.*, 2004a). As

expected, nicastrin interacts with Aph-1, but PS1 NTF was found to interact with Pen-2 as well as with PS1 CTF, and nicastrin and Aph-1 together can also interact with PS1 CTF. Other studies have confirmed the PS1 NTF/Pen-2 interaction and pinpointing transmembrane domain 4 of PS1 as the site of contact with Pen-2 (Kim and Sisodia, 2005; Watanabe *et al.*, 2005). These findings have led to the model for the γ-secretase complex shown in Fig. 4.

The transmembrane domain (TMD) of nicastrin is required for complex assembly. Swapping in a different transmembrane domain prevents incorporation of nicastrin, with the N-terminal region being especially important (Capell *et al.*, 2003). The function, if any, of the very short cytosolic tail of nicastrin is unclear, but the large ectodomain has been elegantly and rigorously shown to play an essential role in substrate recognition (see below) (Shah *et al.*, 2005). The specific biochemical role of the small hairpin Pen-2 protein is unknown, but the seven-transmembrane Aph-1 is thought to be a scaffolding protein for the rest of the complex (Takasugi *et al.*, 2003). The reported topologies of Aph-1 and Pen-2 (Crystal *et al.*, 2003; Morais *et al.*, 2003) are as shown in Fig. 2. As mentioned above, the topology of presenilin has recently been reinvestigated and shown to contain nine transmembrane domains (Laudon *et al.*, 2005; Oh and Turner, 2005). The final three transmembrane segments had been difficult to confirm, one reason apparently being their interdependence. TMD7 is a rather short hydrophobic domain, with one of the conserved aspartates in the middle. Incorporation of this TMD has been shown to require TMD8 (Oh and Turner, 2005). This concept of more hydrophobic regions pulling in less hydrophobic regions is emerging as a theme in membrane protein insertion and folding (Mackinnon, 2005). Another study suggested that presenilin forms a ring-like structure (Annaert *et al.*, 2001), and this notion has received recent support. First, mutagenesis showed that certain residues in TMD1 are critical for
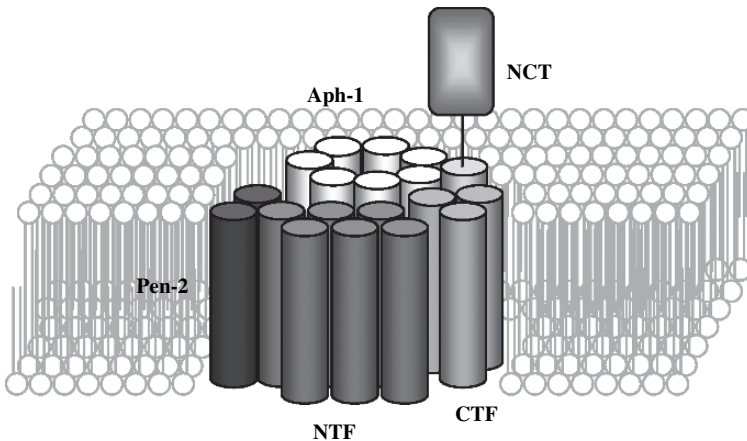

Figure 4. Model for how the components of γ-secretase are arranged within the active protease complex (modified from Fraering *et al.*, 2004a)

γ-secretase activity (Brunkan *et al.*, 2005), and second, a cysteine in TMD1 of the PS1 NTF can be chemically crosslinked to either of two cysteines found in TMD8 of the PS1 CTF (Kornilova *et al.*, 2006). Most of the large lumenal/extracellular loop between TMD6 and TMD7, a region that is poorly conserved, is not essential for proteolytic function of presenilin (Saura *et al.*, 2000). However, a highly conserved hydrophobic region in this loop includes the site of presenilin endoproteolysis (Podlisny *et al.*, 1997), and harbors a domain (including Tyr-288) that is critical for overproduction of Aβ42 (Laudon *et al.*, 2004). The C-terminus of presenilin is also essential for function (Tomita *et al.*, 1999). This region has been shown to contain an ER-retention sequence; once assembled with other γ-secretase members, this region apparently becomes folded into the complex, allowing transport to the Golgi and beyond (Kaether *et al.*, 2004). Evidence specifically suggests the presenilin C-terminus may interact with the nicastrin transmembrane domain (Kaether *et al.*, 2004).

5.   SUBSTRATE-PROTEASE INTERACTIONS

Among the more intriguing questions about the entire emerging family of intramembrane-cleaving proteases is how they handle substrates and cleave their TMDs in at least two locations (γ and ε, see Fig. 1). Because it presumably contains water and uses hydrophilic residues, the membrane-embedded active site should be sequestered from the hydrophobic environment of the surrounding lipid tails. Thus, the active site might be envisioned to be part of a pore or channel that could allow entry of water (Wolfe *et al.*, 1999a). However, the substrate passes through the membrane and cannot enter such a pore or channel directly; docking on the outer surface of the protease, with lateral gating to bring the substrate into the internal active site, might be required (Wolfe *et al.*, 1999a). Initial evidence for such a mechanism came from isolation of the γ-secretase complex with an immobilized transition-state analogue inhibitor (Esler *et al.*, 2002). Detergent-solubilized membranes from human HeLa cells were passed through this affinity matrix, resulting in copurification of γ-secretase complex members and an endogenous membrane-bound APP stub found in HeLa cells. This stub results from alternative processing of APP by α-secretases, and like the stub produced by β-secretase, it is also a γ-secretase substrate. Thus, an endogenous substrate copurified with the γ-secretase complex while the protease active site was blocked by the immobilized transition-state analogue inhibitor, suggesting the existence of a separate substrate binding site. Substrate bound to this special type of exosite, dubbed the "docking site", could copurify without being subject to proteolysis.

Designed peptides based on the transmembrane domain of APP and constrained in a helical conformation can potently inhibit γ-secretase, apparently by interacting with this docking site (Das *et al.*, 2003). Conversion of these helical peptide inhibitors into affinity labeling reagents led to the localization of the substrate docking site to the presenilin NTF/CTF interface (Kornilova *et al.*, 2005). Transition-state analogue inhibitors also bind directly to the NTF/CTF interface,

but at a site distinct from that of helical peptide inhibitors. These findings suggest a pathway for γ-secretase substrate from docking site to active site: upon binding to the outer surface of presenilin at the NTF/CTF interface, the substrate can pass, either in whole or in part, between these two presenilin subunits to access the internal active site (Fig. 5). Interestingly, extension of a ten-residue helical peptide inhibitor by just three additional residues resulted in a potent inhibitor (Bihel *et al.*, 2004) apparently capable of binding both docking site and active site (Kornilova *et al.*, 2005), suggesting that these two substrate binding sites are relatively close.

Up until recently, all the action seemed to be taking place on presenilin. However, an elegant study has demonstrated that nicastrin also plays a critical role in substrate recognition (Shah *et al.*, 2005). The ectodomain of nicastrin bears sequence resemblance to aminopeptidases, although certain catalytic residues are not conserved. Nevertheless, nicastrin recognizes the N-terminus of γ-secretase substrates derived from APP and Notch, and mutation of the aminopeptidase-like domain prevents this interaction. One conserved glutamate is especially important, probably because this residue forms an ion pair with the amino terminus of the substrate. The sequence of the substrate N-terminus is apparently not critical for the interaction, but a free amino group is. Indeed, simple formylation of the substrate N-terminus is enough to prevent effective substrate interaction and proteolytic processing. Thus, nicastrin
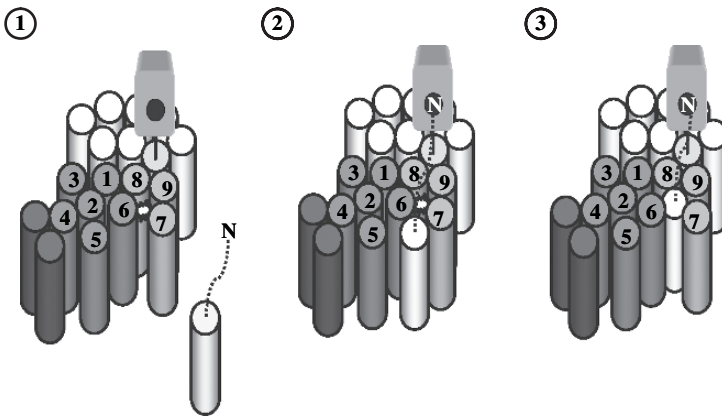

Figure 5. Model for the γ-secretase complex and its interaction with substrate. The transmembrane region of the substrate initially docks at the presenilin NTF/CTF interface, while the N-terminus of the substrate interacts with the nicastrin ectodomain. The substrate, either in whole (as depicted) or in part, then accesses the internal active site, which contains water and two aspartates. Interaction of the substrate N-terminus with the nicastrin ectodomain may facilitate binding of the substrate transmembrane domain to the docking site and/or movement into the active site. The γ-secretase complex is drawn to take into account the following: (1) dissociation into partial complexes using the detergent DDM, (2) the initial formation of nicastrin and Aph-1 as a subcomplex, (3) the interaction of the C-terminus of presenilin with the nicastrin TMD, (4) the interaction between Pen-2 and the presenilin TMD4, and (5) the proximity between presenilin TMD1 and TMD8

can be thought of as a kind of gatekeeper for the γ-secretase complex: type I membrane proteins that have not shed their ectodomains cannot interact properly with nicastrin and do not gain access to the active site (Fig. 5).

6.   γ-SECRETASE AS A THERAPEUTIC TARGET

Although γ-secretase has in many ways been an attractive target for Alzheimer therapeutics, interference with Notch processing and signaling may lead to toxicities that preclude clinical use of inhibitors of this protease. Long-term treatment with γ-secretase inhibitors causes severe gastrointestinal toxicity and interferes with the maturation of B- and T-lymphocytes in mice, effects that are indeed due to inhibition of Notch processing and signaling (Searfoss *et al.*, 2003; Wong *et al.*, 2004). Nevertheless, one γ-secretase inhibitor, LY450139, is currently in phase II clinical trials in the U.S. (Siemers *et al.*, 2006) (Fig. 6). Compounds in this structural class bind to presenilin (Seiffert *et al.*, 2000), and the binding site appears to overlap somewhat with both active site and docking site (Kornilova *et al.*, 2005; Kornilova *et al.*, 2003). While this compound shows little or no selectivity for APP vis-à-vis Notch, the hope is that a therapeutic window exists in which Aβ can be lowered enough to prevent the formation of toxic aggregates while allowing sufficient Notch signaling to avoid toxicities.

Compounds that can modulate the enzyme to alter or block Aβ production with little or no effect on Notch would bypass this potential roadblock to therapeutics. Recent studies suggest that the protease complex contains allosteric binding sites that can alter substrate selectivity and the sites of substrate proteolysis. Certain non-steroidal anti-inflammatory drugs (NSAIDs; e.g. ibuprofen, indomethacin, and sulindac sulfide) can reduce the production of the highly aggregation-prone Aβ42 peptide and increase a 38-residue form of Aβ, a pharmacological property independent of cyclooxygenase inhibition (Weggen *et al.*, 2001). The alteration of the proteolytic cleavage site is observed with isolated or purified γ-secretase (Fraering *et al.*, 2004b; Weggen *et al.*, 2003), indicating that the compounds can interact directly with the protease complex to exert these effects. Enzyme kinetic studies and displacement experiments suggest the selective NSAIDs can be noncompetitive with respect to APP substrate and to a transition-state analogue inhibitor,
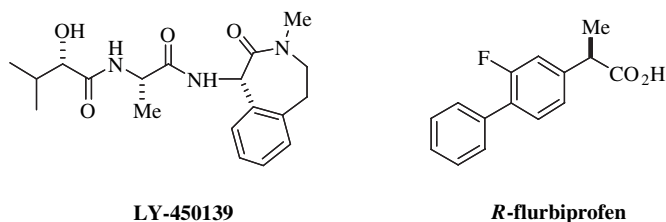

LY-450139   ***R*-flurbiprofen**

Figure 6. γ-Secretase inhibitor (LY-450139) and modulator (R-flurbiprofen) currently in clinical trials for the treatment of Alzheimer's disease

suggesting interaction with a site distinct from the active site (Beher *et al.*, 2004). The site of cleavage within the Notch transmembrane domain is similarly affected, but this subtle change does not inhibit the release of the intracellular domain and thus does not affect Notch signaling (Okochi *et al.*, 2006). For this reason, these agents may be safer as Alzheimer therapeutics than inhibitors that block the active site or the docking site. Indeed, one compound, R-flurbiprofen (Flurizan, Fig. 6), has recently advanced to Phase III clinical trials. Surprisingly, the site of proteolytic cleavage by SPP can also be modulated by the same NSAIDs that affect γ-secretase, suggesting that presenilin is the site of NSAID binding within the γ-secretase complex and that SPP and presenilin share a conserved drug binding site for allosteric modulation of substrate cleavage sites (Sato *et al.*, 2006).

Another type of allosteric modulator are compounds that resemble kinase inhibitors and interact with a nucleotide binding site on the γ-secretase complex. The discovery that ATP can increase Aβ production in membrane preparations prompted the testing of a variety of compounds that interact with ATP binding sites on other proteins (Netzer *et al.*, 2003). In this focused screen, the Abl kinase inhibitor Gleevec emerged as a selective inhibitor of Aβ production in cells without affecting the proteolysis of Notch. In light of these findings, ATP and other nucleotides were tested for effects on purified γ-secretase preparations and found to selectively increase the proteolytic processing of a purified recombinant APP-based substrate without affecting the proteolysis of a Notch counterpart (Fraering *et al.*, 2005). Furthermore, certain compounds known to interact with ATP binding sites were found to selectively inhibit APP processing vis-à-vis Notch in purified protease preparations. The γ-secretase complex could be pulled down with beads containing immobilized ATP, and the presenilin-1 CTF was specifically photolabeled by 8-azido-ATP. This labeling was not blocked by a transition-state analogue inhibitor or by the recombinant APP- and Notch-based substrates; however, the APP-selective inhibitors could prevent photolabeling by 8-azido-ATP. Taken together, these results suggest that the γ-secretase complex contains a nucleotide binding site, to which the presenilin-1 CTF is at least a contributor, and that this site allows allosteric regulation of γ-secretase processing of APP with respect to Notch. Whether this regulation is physiologically important is unclear, but the pharmacological relevance is profound and may lead to new therapeutic candidates for Alzheimer's disease. This hope is tempered by the fact that γ-secretase cleaves numerous other type I membrane protein stubs that result from ectodomain shedding. Agents selective for APP versus Notch may reveal new long-term toxicities due to blocking proteolysis of these other substrates, toxicities masked by the severe Notch-related effects with nonselective inhibitors.

7.   CONCLUSIONS

γ-Secretase is a founding member of a new class of membrane-embedded proteases that process the transmembrane domains of their substrates (Wolfe and Kopan, 2004). These enzymes also include (1) the site 2 protease (S2P) family,

putative metalloproteases responsible for cholesterol and fatty acid biosynthesis in metazoans and mating factor signaling in bacteria, (2) the rhomboid family, serine proteases involved in growth factor signaling, mitochondrial matrix remodeling, and parasite invasion, and (3) the SPP family of presenilin homologs, exemplified by signal peptide peptidase, which processes remnant signal peptides produced by signal peptidase and plays a role in immune surveillance and maturation of certain hepatitis C core proteins. Discovery of membrane proteins responsible for these proteolytic activities has led to some degree of understanding of their mechanisms and how these proteases interact with substrates. However, intimate understanding of enzymatic mechanisms, including direct evidence for the involvement of the putative catalytic residues, will likely require detailed structural information. Indeed, this is considered by many to be the next major goal in this fascinating field of investigation. High resolution structures along with further molecular and biochemical studies will provide mechanistic insight and a path to structure-based drug design.

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CHAPTER 7

γ-SECRETASE MEDIATED PROTEOLYSIS: AT THE CUTTING EDGE OF NOTCH SIGNALING

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Abstract: Notch proteins are evolutionary conserved transmembrane receptors used by metazoans to direct cell fate decisions, proliferation, differentiation and cell death at all stages of development, including self-renewing adult tissues. Notch signaling is a well-established example of a pathway that is mediated by Regulated Intramembrane Proteolysis (RIP). Upon binding of ligand, the Notch receptor undergoes successive proteolytic cleavages – an ectodomain shedding cleavage followed by intramembrane proteolysis by γ-secretase. This process releases the Notch intracellular domain, which translocates to the nucleus to activate its target genes. Deciphering the proteolytic mechanism for Notch activation relied on the convergence of previously independent fields of research, revealing that the Notch receptor resembled another Type I membrane protein, the amyloid-β precursor protein, in that both are proteolytically cleaved within their transmembrane domains (TMDs) by the same protease, γ-secretase, whose catalytic center resided in the protein Presenilin. Intramembrane proteolysis has continued to emerge as an exciting research area in cell biology. Recent studies on γ-secretase function have begun to reveal the molecular details involved in ectodomain shedding and intramembrane cleavage events as well as the importance of endocytosis and endosomal sorting as key regulators of γ-secretase cleavage of Notch

Keywords: Notch, γ-secretase, RIP, ectodomain shedding, endocytosis

1.   INTRODUCTION

The Notch pathway constitutes a short-range communication transducer that is utilized across a wide variety of tissues throughout development and in the adult. Depending on dose and context, Notch signaling can regulate many fundamental aspects of multicellular life: proliferation, stem cell and stem cell niche maintenance, cell fate specification, differentiation and cell death. Accordingly, misregulation or misexpression of Notch signaling components can cause several human disorders

and can either promote or suppress cancer (reviewed in Callahan and Egan, 2004; Collins *et al.*, 2004; Gridley, 2003; Nickoloff *et al.*, 2003; Radtke *et al.*, 2006).

The Notch receptors (Notch 1-4 in mice and humans) are large single pass Type I transmembrane proteins (Fig. 1A). The Notch extracellular domain is composed of 30–36 epidermal growth factor (EGF)-like repeats, three cysteine-rich Lin12-Notch repeats (LNR) and the heterodimerization domain (HD). This is followed by the single predicted transmembrane domain (TMD) and the cytoplasmic tail, which contains an N-terminal RAM (RBPjκ associated molecule) domain, nuclear local-izing sequences (NLS), seven ankyrin repeats (ANK domain), and the C-terminal PEST domain. A few other domains have been proposed to reside at the C-terminus, including a trans-activation domain (TAD) and a putative second RBPjκ inter-action domain (PPD). The majority of Notch protein found at the cell surface in vertebrates has been processed at cleavage site 1 (S1) by a furin-like convertase in the trans-Golgi compartment and therefore targeted to the cell surface as a heterodimer held together by non-covalent interactions within the HD (Fig. 1) (Malecki *et al.*, 2006; Sanchez-Irizarry *et al.*, 2004; Vardar *et al.*, 2003). The receptors are then activated upon the binding of ligands (most belong to the Delta and Serrate/Jagged/Lag2 (DSL) family; (Nye and Kopan, 1995)) that are expressed on neighboring cells.

Over the past decade, the efforts of many groups have led to the discovery that a novel signaling paradigm, Regulated Intramembrane Proteolysis (RIP) (Brown *et al.*, 2000), controls Notch receptor activation (Fig. 1B). In this signaling mechanism, ligand regulated proteolysis releases a functional signaling fragment from the intact precursor. As a result of RIP, a receptor fragment responds *directly* to stimuli instead of propagating a signal through a cascade of secondary messengers typically associated with other major signaling pathways. RIP is known as a two-step process in which the second involves intramembrane-cleaving proteases (I-CLiPs) that catalyze peptide bond hydrolysis within the TMDs of their substrates. In the case of Notch, ligand binding to the Notch receptor is coupled to ligand endocytosis (Borgne *et al.*, 2005) which is thought to convey the force necessary to expose site 2 (S2) to metalloproteases, most likely ADAM family members, thereby shedding the Notch ectodomain and creating a membrane-tethered intermediate called NEXT (for Notch extracellular truncation). This is immedi-ately followed by proteolysis within the TMD (at cleavage sites S3 and S4) by γ-secretase, a novel multi-component I-CLiP (Fortini, 2002; Mumm *et al.*, 2000). The freed Notch intracellular domain (NICD) subsequently translocates to the nucleus where it interacts with the DNA-binding protein CSL (CBF1/RBPjκ in vertebrates, Su(H) in *Drosophila*, Lag-1 in *Caenorhabditis elegans*) thereby leading to the upregulation of downstream target genes (Lubman *et al.*, 2004) (Fig. 1B).

In the first part of this chapter, we provide a historical review of how RIP and γ-secretase became recognized as the underlying mechanism of Notch activation. In the second part, we will summarize our current understanding of the mechanism and regulation of γ-secretase cleavage of Notch.
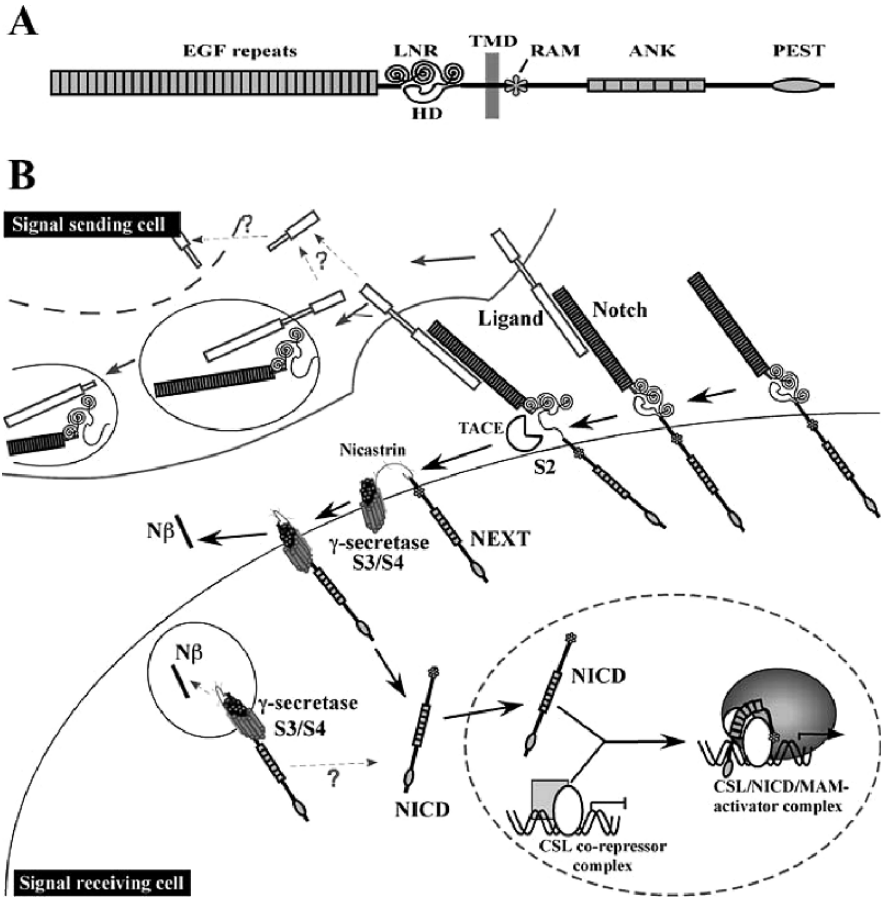
Figure 1. Overview of the Notch signaling pathway. A. Domain organization of the Notch receptor. See text for details. B. Notch signaling is mediated by Regulated Intramembrane Proteolysis. The mature receptor is produced after proteolytic cleavage by furin at Site 1 (S1) and thereafter targeted to the cell surface as a heterodimer held together by non-covalent interactions. Ligands on the neighboring cells bind to the extracellular domain of Notch. Ligand endocytosis leads to a conformational change, exposing the cleavage site S2 to metalloproteases like TACE. Juxtamembrane cleavage at S2 releases the Notch extracellular domain and generates the membrane-anchored NEXT (Notch extracellular truncation) fragment. Nicastrin recognizes and transfers NEXT to the active site of the γ-secretase complex. γ-secretase cleaves the Notch TMD (sites S3 and S4) to release NICD (Notch intracellular domain) and Nβ peptides. NICD enters the nucleus and binds CSL, converting the transcriptional repressor complex into an activator complex. γ-secretase cleavage occurs at the cell surface or NEXT may be endocytosed and cleaved in an endosomal compartment. γ-secretase-mediated cleavage of ligand has also been observed although the physiological relevance of this cleavage is not yet clear

2.   INTRAMEMBRANE PROTEOLYSIS OF NOTCH: A HISTORICAL PERSPECTIVE

2.1.   Notch as a Dual Address Protein

Hints that the Notch receptor was a "dual address" protein (proteins which perform functions at two separate subcellular locations) came from early structure/function analyses, which suggested that the extracellular and intracellular domains of the Notch receptor had distinct activities (for a review, see Greenwald, 1994; Greenwald, 1998; Kimble *et al.*, 1998). Similar to most cell surface receptors, the extracellular domain of Notch interacted with ligand and regulated signaling by preventing activity in the absence of ligand binding, while the intracellular domain was found to have signal-transducing capabilities. However, unlike other surface receptors, several studies suggested that the intracellular domain might directly function as a transcriptional cofactor within the nucleus. Truncated, intracellular Notch constructs lacking a membrane tether (referred to as NICD, Notch-intra or N[IC]) produced constitutive gain-of-function phenotypes when expressed in flies and worms lacking endogenous Notch/*lin-12* activity (Lieber *et al.*, 1993; Roehl and Kimble, 1993; Struhl *et al.*, 1993) and were localized in the nucleus (Fortini *et al.*, 1993; Lieber *et al.*, 1993; Struhl *et al.*, 1993). Putative nuclear localizing signals (NLS) were identified in the cytoplasmic sequence of Notch homologs (Stifani *et al.*, 1992). Altogether, these studies strongly suggested that the NICD fragment could mimic Notch activity but did not establish whether the activity occurred at the cell surface or in the nucleus. Deleting the NLS sequence from Notch1 ICD abrogated its activity in a myogenic assay while fusing a heterologous NLS sequence restored the ability for nuclear entry as well as the activity (Kopan *et al.*, 1994); these observations supported a mechanistic model based on nuclear activity. This model was strengthened when NICD was shown to physically associate with the DNA-binding protein RBPjκ in a yeast two hybrid assay (Tamura *et al.*, 1995) and in nuclear extracts (Jarriault *et al.*, 1995) as well as to help activate expression of specific target genes (Chen *et al.*, 1997; Eastman *et al.*, 1997; Jarriault *et al.*, 1995).

Support for RIP as the Notch activation mechanism was also obtained from the characterization of Notch chromosomal translocations and viral integration hot-spots associated with neoplastic transformation (Ellisen *et al.*, 1991; Jhappan *et al.*, 1992; Robbins *et al.*, 1992). Based on the presumed gene products, deletion constructs were made that had the majority of the extracellular domain removed (collectively referred to as NΔE). NΔE constructs differ from NICD in that the signal peptide and TMD have been retained thereby keeping the intracellular domain tethered to the plasma membrane. When ectopically expressed, NΔE produced activated Notch phenotypes in frogs (Coffman *et al.*, 1993) and in flies (Fortini *et al.*, 1993; Rebay *et al.*, 1993) demonstrating that like NICD, NΔE operated as a constitutively active protein. In the next few years cell culture-based experiments further confirmed the constitutive activity of NΔE and NICD moieties; both Notch constructs similarly activated target reporter constructs, inhibited myogenic and

neurogenic differentiation, and impacted T cell development (Aster *et al.*, 1994; Jarriault *et al.*, 1995; Kopan *et al.*, 1994; Nye *et al.*, 1994; Pear *et al.*, 1996; Shawber *et al.*, 1996). Based on these observations, an activation mechanism for Notch was proposed that would explain why both NICD and NΔE act equivalently: cleavage of the Notch receptor released a fragment that could enter and function in the nucleus (Kopan *et al.*, 1994; Lieber *et al.*, 1993; Struhl *et al.*, 1993). Although nuclear Notch immunoreactivity was observed in rat retinas (Ahmad *et al.*, 1995) and human cervical tissue (Zagouras *et al.*, 1995), this proteolysis-based mechanistic model was rejected since endogenous NICD was not detected in the nuclei of developing *Drosophila* embryos and worms in tissues where Notch signaling was known to be active (Aster *et al.*, 1997; Crittenden *et al.*, 1994; Johansen *et al.*, 1989; Kooh *et al.*, 1993).

The proteolysis model gained support when it was demonstrated that membrane tethered NΔE constructs could be converted to the NICD fragment via proteolysis in transfected mammalian cells and in frog ectoderms leading to a refined model that suggested that ligand acted to antagonize a proteolysis-inhibiting domain (the LNR), permitting an endogenous protease to liberate NICD (Kopan *et al.*, 1996). Schroeter *et al.*, (1998) went on to show that N1ΔE is cleaved between Gly 1743 and Val 1744 at a site (S3) located near the cytoplasmic side of the lipid bilayer (Fig. 2) and that this cleavage was triggered by ligand binding to the full length Notch 1 protein. Single amino acid substitutions at V1744 reduced S3 processing and concomitantly reduced the signaling activity of N1ΔE while having no effect on the signaling efficacy of cleavage-independent N1ICD V1744 mutant proteins.

Intramembrane Proteolysis of Notch1:

Figure 2. Dual intramembranous cleavage of Notch transmembrane domain: Initial cleavage of the Notch transmembrane domain by γ-secretase occurs close to the cytosolic side at a site termed S3, and releases NICD. The corresponding cleavage in the transmembrane domain of Amyloid precursor protein (APP), termed ε, can occur at more than one site in the vicinity. Similarly, it is possible that S3 cleavage may also occur at other neighboring residues, although cleavage between Gly1743 and Val1744 is the predominant product that has been demonstrated to have physiological significance. Subsequent intramembranous cleavages occur at the center of the transmembrane domain at a site termed S4. Although S4 cleavage predominantly occurs between Ala1731 and Ala1732 (arrow), minor products are observed from cleavages at adjacent sites (underlined)

In addition, the ability of ligand binding to promote S3 processing of full length Notch was also dependent on V1744 (Schroeter *et al.*, 1998). The inability to detect endogenous nuclear NICD was suggested to reflect NICD levels lower than the limit of detection, and indeed Schroeter *et al.* could demonstrate target activation occurring at NICD levels undetectable even with antibodies directed to a multimerized tag. Concurrent with these studies, ligand-dependent nuclear translocation of Notch::GAL4VP16 fusion protein, presumably via proteolysis, was demonstrated to occur *in vivo* in *Drosophila* (Lecourtois and Schweisguth, 1998; Struhl and Adachi, 1998). In parallel studies it was shown that modulation in the expression level of the Notch ligand Delta resulted in corresponding changes in NICD production (Kidd *et al.*, 1998). The requirement for Notch1 cleavage was demonstrated unequivocally in a mouse model: a Notch1 allele with a single amino acid substitution at the S3 site (V1744G) was homologously knocked-in into the Notch1 locus. Mice homozygous for this hypomorphic V1744G allele exhibited an embryonic lethal phenotype (Huppert *et al.*, 2000) strikingly similar to that of Notch1 null mice (Conlon *et al.*, 1995; Swiatek *et al.*, 1994). Finally, the development of antibodies to the amino terminus of cleaved Notch1 allowed for the development of sensitive protocols permitting for the first time to detect the endogenous NICD fragment in cells undergoing active Notch signaling (Cheng *et al.*, 2003; Huppert *et al.*, 2005; Morimoto *et al.*, 2005; Tokunaga *et al.*, 2004), thereby eliminating the last obstacle for the wide acceptance of RIP as the mechanistic basis for Notch activation.

It is now well established that NICD functions in the nucleus and a crystal structure describing the Notch/CSL nuclear complexes from humans and worms provides detail at the atomic level (Barrick and Kopan, 2006; Nam *et al.*, 2006; Wilson and Kovall, 2006). In the absence of NICD, CSL proteins complex with ubiquitous co-repressor proteins such as SKIP, SMRT (Kao *et al.*, 1998) and MINT (Kuroda *et al.*, 2003) to repress transcription of target genes. Once it reaches the nucleus, RAM domain binding to CSL is thought to trigger an allosteric change that may facilitate shedding of transcriptional repressors (Barrick and Kopan, 2006). Interaction with CSL is mediated through a WFP motif in the RAM domain with a small contribution from the ANK domain (Lubman *et al.*, 2006; Tamura *et al.*, 1995; Tani *et al.*, 2001). The ANK/CSL interface is then recognized by Mastermind/Lag-3 protein (Nam *et al.*, 2006; Petcherski and Kimble, 2000; Wilson and Kovall, 2006), and this tri-protein complex recruits histone acetyltansferases (reviewed in Lubman *et al.*, 2004) and a mediator complex (Fryer *et al.*, 2004) to assemble an active transcription complex on target promoters. During this process, NICD is phosphorylated on its PEST domain and targeted for proteasomal degradation by several E3 ubiquitin ligases, which terminates the Notch signal (Lubman *et al.*, 2004; Tsunematsu *et al.*, 2004) and resets the cell for the next round of signaling.

Most of the early studies in mammalian cells were focused on Notch1 but a biochemical comparison of the four mouse Notch homologs has shown that all four receptors undergo an S3-like cleavage event (Mizutani *et al.*, 2001; Saxena *et al.*, 2001) supporting the idea that intramembrane proteolysis is a

conserved signaling mechanism that is required for CSL-mediated Notch signaling in all metazoans. While proteolysis may be integral to the signaling mechanism of all Notch homologs, they may have distinct activities in the nucleus which may not all involve CSL (Cheng *et al.*, 2001; Gustafsson *et al.*, 2005; Shin *et al.*, 2006). How different Notch homologs acquired specific activities remains to be resolved.

2.2. Notch is Activated by RIP via Presenilin/γ-secretase

As experimental evidence for Notch proteolysis and a direct nuclear function for the NICD fragment was growing in the early 1990s, a key question that arose was the identity of the protease(s) that catalyzed this unusual intramembrane cleavage of the receptor. The answer came from the convergence of previously independent areas of research, revealing that the Notch receptor resembled another Type I membrane protein, the amyloid-β precursor protein (APP), in that both are proteolytically cleaved within their TMDs by the same protease, γ-secretase, whose catalytic center resided in the protein Presenilin (PS).

The γ-secretase enzyme had been the focus of intense efforts by many Alzheimer's disease (AD) researchers (Selkoe and Kopan, 2003). After sequencing amyloid plaque constituents from AD and Down syndrome patients (Masters *et al.*, 1985) and realizing that the amyloidogenic Aβ peptides are derived from APP, γ-secretase activity was proposed to generate the C-terminus of Aß by cleaving at one of several positions (i.e. γ-sites) within the APP TMD. Initially, each cleavage site was thought to reflect a distinct enzymatic activity as γ-secretase cleavage released Aß peptides from 38 to 43 residues long (Murphy *et al.*, 1999). Of these, the longer more aggregation prone Aβ42 fragments are thought to be central to the disease process (the "ß-amyloid hypothesis"). It was eventually demonstrated that like Notch, the N-terminus of Aβ is generated when the APP ectodomain is shed by α- or β-secretase (see Chapter 6), producing membrane-associated C-terminal fragments that have different amino termini based on which enzyme was involved in shedding.

The search for genetic determinants of AD resulted in the discovery of PS (Group, 1995; Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995; Sherrington *et al.*, 1995). Mutations in PS1, PS2 and APP are autosomal dominant, causing familial AD (FAD; see chapter 6). Key evidence demonstrating that the PS proteins were the long-sought catalytic component of γ-secretase included inhibitor studies characterizing the pharmacological properties of γ-secretase as an aspartyl protease (Shearman *et al.*, 2000; Wolfe *et al.*, 1999a). PS lack the classic D(T/S)G motif of an aspartyl protease but a closer look at the amino acid sequence revealed two highly conserved aspartyl residues residing in TMDs 6 and 7 (Asp257 and Asp385 in PS1, respectively). Substitution of either of these two aspartyl residues with an alanine led to a loss of γ-secretase activity (measured by APP cleavage), supporting the idea that PS represented a novel class of aspartyl proteases in which the catalytic aspartyl residues are embedded in the membrane (Haass and Steiner, 2002; Ray *et al.*, 1999b; Wolfe *et al.*, 1999b). Subsequent biochemical studies demonstrated

that solubilized γ-secretase activity co-eluted with heterodimeric PS1, and γ-secretase activity co-immunoprecipitated with PS1 from the soluble extract (Li *et al.*, 2000a). Importantly, inhibitors based on transition state analogues, which are expected to bind the active site of γ-secretase, were independently shown to bind directly to PS proteins (Esler *et al.*, 2000; Li *et al.*, 2000b; Seiffert *et al.*, 2000).

A role of PS in Notch signaling was first suggested when loss of Sel-12, a presenilin homolog from *C. elegans*, was shown to suppress an activating point mutation in the Notch homolog Lin-12 (Levitan and Greenwald, 1995). Sel-12/PS activity was mapped at or upstream of S3 cleavage and NICD release since loss-of-function mutations in *sel-12* failed to suppress the constitutive activity of NICD, but were able to suppress the activity of dominant gain-of-function mutations in the Lin-12 LNR domain (Levitan and Greenwald, 1998; Levitan *et al.*, 2001; Li and Greenwald, 1997). A physical association between Notch and PS, as well as the ability of aspartyl mutant forms of PS1 to bind Notch, co-translocate with it to the cell surface but block its proteolysis led to the suggestion that PS were involved in Notch cleavage (Ray *et al.*, 1999a; Ray *et al.*, 1999b). Consistent with the findings that Aβ production is reduced in the absence of PS1 (De Strooper *et al.*, 1998), S3 cleavage of Notch is dramatically reduced in the absence of PS1 (De Strooper *et al.*, 1999). In cells that lack both PS proteins, no γ-secretase activity is observed and NΔE is no longer able to signal (De Strooper *et al.*, 1999; Herreman *et al.*, 2000; Song *et al.*, 1999; Zhang *et al.*, 2000). This hypothesis became dogma when flies, mice and worms lacking all PS alleles displayed Notch-like phenotypes (De Strooper *et al.*, 1999; Donoviel *et al.*, 1999; Guo *et al.*, 1999; Herreman *et al.*, 1999; Li and Greenwald, 1997; Song *et al.*, 1999; Struhl and Greenwald, 1999; Westlund *et al.*, 1999; Ye and Fortini, 1999). Consistent with this observation, γ-secretase inhibition blocks Notch activity in organ cultures (Doerfler *et al.*, 2001; Hadland *et al.*, 2001) and model organisms (Geling *et al.*, 2002; Micchelli *et al.*, 2003). The latter realization underlies both the aversion towards the use of γ-secretase inhibitors in chronic treatment of AD (Kopan and Ilagan, 2004) and their resurgence as drugs of choice for treatment in several types of cancer involving Notch (Dontu *et al.*, 2004; Fan *et al.*, 2006; Nam *et al.*, 2002). As detailed in Chapter 6, it is now clear that cells contain several γ-secretase complexes each consisting of at least 4 proteins: presenilin (1 or 2 but never both), Nicastrin, APH-1 (1a or 1b or 1c), and PEN-2. Several accessory proteins have been identified (e.g. CD147, TMP21); however, whether any complexes have distinct properties *in vivo* as it relates to Notch proteolysis is unclear. It is important to note in this context that many critical Notch dependent processes in the vascular, neural, urogenital, and cutaneous organs still occur normally in mice lacking PS1 (see images of PS1 deficient mice in Qian *et al.*, 1998; Wong *et al.*, 1997). The cause of death of these mice is most likely due to rib defects and the subsequent suffocation after birth. Indeed, mice with less than 1% of PS1 protein display severe defects only in the somite derivatives posterior to the rib cage and are otherwise viable (Mastrangelo *et al.*, 2005; Rozmahel *et al.*, 2002a; Rozmahel *et al.*, 2002b). These data strongly argue that PS2 complexes are sufficient to provide all early functions of γ-secretase

and that they fail only in the presomitic mesoderm (Mastrangelo *et al.*, 2005) where a PS-dependent activity unrelated to γ-secretase plays an important role (Huppert *et al.*, 2005).

3. MECHANISM AND REGULATION OF THE γ-SECRETASE CLEAVAGE OF NOTCH

3.1. The Notch Ectodomain Regulates Proteolysis

Genetic analysis of Notch mutants and structure/function analyses of Notch proteins (e.g. NΔE-like molecules) in flies and worms suggested that the extracellular domain has three functions. First, to bind ligand in *trans* (via EGF repeats 11-12, (de Celis *et al.*, 1993; Lei *et al.*, 2003)). A second region binds ligand in *cis* (via EGF repeats 24-29, a region also known as the Abruptex domain, (de Celis and Bray, 2000; Micchelli *et al.*, 1997; Portin, 1975)). The *cis* interactions are antagonistic, while the *trans* interactions are agonistic (see below). Finally, the juxtamembrane region functions to inhibit Notch proteolysis in the absence of ligand (Greenwald, 1994; Kimble *et al.*, 1998; Kopan *et al.*, 1996). Based on genetic analyses of gain-of-function alleles, this negative control region includes LNR as well as the region between LNR and the TMD (Greenwald, 1994; Kimble *et al.*, 1998; Lieber *et al.*, 1993), more recently termed the HD region (Fig. 1A). Cancer causing mutations in humans map to this region in almost 50% of sporadic human T-ALL (Weng *et al.*, 2004).

The Notch activation cycle begins with ligand binding; our current understanding of the biochemistry of this process stems from two observations – the role of endocytosis in activation (Parks *et al.*, 2000) and the discovery that ectodomain shedding acts as a regulatory point in Notch signal transduction (Brou *et al.*, 2000; Mumm *et al.*, 2000). Biochemical analyses of Notch molecules activated by mutations in the LNR/HD region revealed the appearance of a novel proteolytic fragment (Brou *et al.*, 2000; Mumm *et al.*, 2000) that was not observed with inactive molecules and that migrated slower than the NICD fragment during SDS-PAGE analyses. It was further demonstrated that Notch becomes a substrate to an extracellular protease cleaving at a second site (S2), resulting in a membrane-tethered intermediate called NEXT (for Notch extracellular truncation) (Fig. 1B). Inhibition of S3 cleavage by mutation in the S3 site (Mumm *et al.*, 2000), γ-secretase inhibitors (Mumm *et al.*, 2000), or loss of PS (Lieber *et al.*, 2002) leads to the accumulation of NEXT fragments, indicating that once formed it is rapidly converted to NICD via cleavage at S3. S2 cleavage occurs between Ala1710 and Val1711 residues of mouse Notch1 (Mumm *et al.*, 2000), approximately 12 amino acids outside the TMD, via the action of a metalloprotease as it is sensitive to zinc chelation (Mumm *et al.*, 2000). ADAM metalloproteases Kuzbanian/ADAM10 and/or TACE (tumor necrosis α (TNFα) converting enzyme /ADAM17 (Brou *et al.*, 2000; Lieber *et al.*, 2002; Wen *et al.*, 1997)) are the leading candidates for the S2 enzymes. Indeed, the S2 cleavage site is consistent in sequence and in its location relative to the membrane with other

known TACE substrates. Interestingly, although TACE is able to cleave Notch substrates *in vitro* (Brou *et al.*, 2000), the phenotype of ADAM17/TACE knockout mice is not as severe as that of Notch null mice (Peschon *et al.*, 1998). Conversely, loss of Kuzbanian/ADAM10/Sup-17 function produced Notch null-like phenotypes in all metazoans (Hartmann *et al.*, 2002; Lieber *et al.*, 2002; Rooke and Xu, 1998; Sotillos *et al.*, 1997; Wen *et al.*, 1997) yet it fails to cleave Notch *in vitro* (Brou *et al.*, 2000). The role of other metalloproteases in S2 cleavage is still a matter of active investigation. Notably, Kuz and TACE, which are also known as α-secretase, catalyze cleavage of the APP extracellular domain as well as that of many other type I proteins, several of which are also cleaved by γ-secretase.

Initially, it was thought that a change in the oligomerization status of Notch regulated receptor activation/proteolysis. Genetic studies led to speculation that ligand binding could initially promote dimerization to activate the receptor (Greenwald, 1994; Greenwald, 1998; Greenwald and Seydoux, 1990; Heitzler and Simpson, 1993). Negative complementation of viable Abruptex alleles (i.e. the embryo die only when two different Notch mutations are present in the same genome (Portin, 1975; Portin and Rantanen, 1990)) also supported the idea that oligomer-ization played some important role in regulating Notch activity. Later models attempting to explain the regulation of Notch proteolysis postulated that oligomers were resistant to proteolysis and that ligand binding generated monomeric Notch molecules (Kopan *et al.*, 1996; Struhl and Adachi, 2000). This model gained strong support from experimental manipulations that measured the signaling efficiency of artificial Notch molecules whose oligomeric state could be modulated by the investigator (Struhl and Adachi, 2000). However, it was later demonstrated that the oligomeric state of surface Notch protein did not correlate with activity; mutations in the LNR/HD regions increase basal activity without altering the oligomeric state of Notch molecules (Vooijs *et al.*, 2004). While it remains to be determined whether ligand binding can change the oligomerization status of bound receptor, a major role for oligomerization in controlling proteolysis appears unlikely.

An alternative model (the dissociation model) proposed that S1 cleavage is critical for activation and that ligand binding somehow dissociates the LNR/HD domain. The first observation in support of the dissociation model is the demonstration that disrupting the interactions within the HD domain by calcium chelation results in Notch1 activation via S3 cleavage (Rand *et al.*, 2000). Urea unfolding analysis of gain-of-function point mutations from T-ALL patients established that many of these mutations destabilize the folding of the HD domain, most likely leading to its dissociation around a pre-existing S1 site (Malecki *et al.*, 2006). Recent exper-iments with over-expressed microfibrillar protein MAGP (Miyamoto *et al.*, 2006) suggest that such dissociation can occur in Notch given the right extracellular environment. However, not all metazoan Notch proteins are cleaved by furin (Kidd and Lieber, 2002), and there is no evidence that MAGP proteins contribute in any way to Notch signaling under physiological conditions. Thus, the dissociation model is best at explaining pathological activation of Notch in T-ALL.

A third model was inspired by the observation that the Notch ectodomain localized to ligand-presenting cells in *Drosophila*, but the Notch intracellular domain was only found in signal receiving cells (Parks *et al.*, 2000). A genetic link between endocytosis and Notch signaling was established by the characterization of *shibire* (*shi*), the Dynamin homolog in *Drosophila*. Dynamin, a pleckstrin homology repeat containing GTPase, is necessary for pinching-off clathrin coated pits from the plasma membrane during endocytosis. *Shi* mutants show strikingly similar phenotypes to Notch loss-of-function alleles during several developmental processes in *Drosophila*. Another endocytosis mutant *bag* encoding the clathrin heavy chain was also isolated as a modifier of Notch signaling in a genetic screen. Coupled with the observation that loss of dynamin abrogated Notch signaling (Parks *et al.*, 2000; Seugnet *et al.*, 1997), the authors proposed that "mechanical strain" generated by receptor trans-endocytosis somehow exposed the S2 site for cleavage (see also Le Borgne and Schweisguth, 2003). Genetic analyses during peripheral nervous system development indicate that truncated NΔE (a ligand-independent molecule that resembles NEXT) is properly processed at S3 in *shi* mutants (Struhl and Adachi, 2000). These data argue that endocytosis is only required for ligand-mediated activation upstream of S3, most likely to facilitate S2 cleavage or dissociation at S1. While in itself unable to resolve these issues, the conformational-change model was born to explain why endocytosis was required. Although it is unclear how force will be transferred from EGF 11-12 to the LNR (NMR analysis of the Notch extracellular domain suggests a semi-rigid structure that is unlikely to transmit a long range allosteric change (Hambleton *et al.*, 2004)), several additional observations makes this model a strong front-runner. First, Notch and Delta expressing cells bind each other in *trans* extremely tightly (Ahimou *et al.*, 2004), suggesting that once formed, the Notch/ligand complex is sufficiently stable to necessitate forces generated by endocytosis to contort the juxtamembrane structure. Second, a 14 amino acid insertion at the S2 site of human Notch1 displaced S2 a short distance away from the LNR/HD. Unlike the HD point mutations, this molecule is as stable as the wild type Notch in up to 3.5M urea, indicating that the LNR/HD structure has not been compromised by the insertion (Malecki *et al.*, 2006). If such a short distance is sufficient to permit ligand independent proteolysis, and thus constitutive activation, the force generated by endocytosis may need to generate only a modest dislocation/distortion in order to uncover S2 (Le Borgne and Schweisguth, 2003). Third, soluble ligands do not activate Notch but immobilized ligands do, consistent with a model in which force, generated this time by Notch endocytosis, acts to expose S2 (Varnum-Finney *et al.*, 2000). Fourth, the ability of ligand to antagonize Notch signaling by binding to the Abruptex regions (EGF repeats 24-30) may do so by buttressing Notch against the forces generated by trans-endocytosis (Mishra-Gorur *et al.*, 2002). Finally, Notch activation via calcium chelation, which is used to support a dissociation model, could also be explained by alterations in the LNR structure (i.e. induction of a conformational change) to permit S2 cleavage.

The structure of the LNR/HD domain solved recently by the Blacklow group resolves many of these ambiguities (S.B., personal communication). The two halves of the HD domain are intimately intertwined into a single protein domain, with the position of the S1 site inferred to be located in a loop far removed from the metalloprotease cleavage site. The three LNR modules (LNR-A, B and C) encircle the HD domain to create an overall structure resembling a three-leafed clover (see Fig. 1A). Importantly, S2 is located in a ß-strand buried within an inaccessible pocket protected by hydrophobic interactions with the LNR-AB linker and a neighboring helix within the HD domain, which is buttressed by extensive interactions with LNR-B. Thus, two protective devices prevent premature cleavage of the receptor in the absence of ligand: direct steric occlusion (by the LNR-AB linker) and global domain stabilization (by interactions with LNR-B). In reporter assays, LNR-A, the AB linker, and LNR-B must all be removed to observe signaling activity, perhaps because the neighboring helix buttressed by LNR-B is still hindering access to S2. Given the deep active site pocket in TACE (Ingram *et al.*, 2006; Maskos *et al.*, 1998; Wasserman *et al.*, 2003), Blacklow's group speculates that not only does the receptor activation mechanism need to forcibly lift at least two of the three LNR repeats, the process must also disengage the stabilizing helix in the HD domain from the strand containing S2 (perhaps by unfolding the helix) to permit entry of the scissile bond at S2 into the TACE active site. Alternatively, another enzyme, with a shallower active site, could mediate cleavage of S2 without further conformational alteration within the HD domain. This structure clearly defines the structure of the "off" state of the receptor, shows that autoinhibition is intrinsic to a single Notch monomer, establishes the requirement for a large scale conformational movement, and provides a molecular logic to support the idea first suggested by Parks and Muskavitch that mechanical force will be needed to expose the metalloprotease cleavage site. The structure also raises perplexing questions about the precise mechanism involved in transferring tensile force along the extracellular domain. Perhaps additional structures will clarify whether the isolated HD assumes a conformation more amenable to cleavage, which can possibly be induced by activating mutations in this region.

It is worth noting here that the requirement for juxtamembrane cleavage prior to intramembrane cleavage appears to be a common feature for most I-CLiPs, with the Rhomboids being the notable exception (Weihofen and Martoglio, 2003). In the case of Notch and other I-CLiP substrates, ligand binding controls juxtamembrane cleavage. Interestingly, regulation of γ-secretase cleavage in RIP can also be accomplished in a ligand-independent manner via alternative splice forms that produce proteins that are either permissive or resistant to TACE cleavage as has been observed for ErbB4 (Sardi *et al.*, 2006). Moreover, γ-secretase is a unique *multiprotein* I-CliP, having recruited Nicastrin to act in a substrate recognition role, as elegantly demonstrated by Shah *et al.* (Shah *et al.*, 2005). Even though all membrane-tethered forms of Notch can interact with PS at the membrane and early in the secretory pathway (Ray *et al.*, 1999a; Ray *et al.*, 1999b), only molecules with a short, free amino-terminus are recognized by Nicastrin, a molecule related

to aminopeptidases (Fagan *et al.*, 2001), which appears to function as a substrate receptor and translocator (from the binding site to the active site). This quirk of γ-secretase could explain why receptor dissociation leads to Notch activation as exposure of the amino-terminus from the prior S1 cleavage may act as S2 cleavage, leading to recognition by Nicastrin, translocation into the active site and subsequent S3 cleavage. Moreover, this requirement would have confounded the analysis of oligomeric, truncated Notch molecules (Struhl and Adachi, 2000) if the oligomeric state, rather than the length, obscured the amino terminus and thus prevented its recognition by Nicastrin. It still remains to be seen whether Nicastrin binding to a free amino-terminus is discriminatory (i.e. exhibiting some substrate specificity) and how Nicastrin coordinates with the other docking sites that map to PS. Another interesting question for future study is how substrate recognition is accomplished in other I-CLiPs.

That S2 cleavage is required for S3 cleavage and NICD release makes it a critical point of regulation for Notch activation. Precisely how ligand binding stimulates S2 cleavage awaits further biochemical and biophysical characterization of inactive versus activated receptors; perhaps the difference involves a switch between two conformational states of HD, or perhaps the force is sufficiently sustained to distort the molecule until S2 is exposed to cleavage. The ability to turn Notch signaling on or off whenever needed for therapeutic or tissue-engineering purposes is a critical skill yet to be achieved; understanding the details of the S2 control switch may prove critical to that end.

3.2. Intramembrane Proteolysis of Notch

The intramembranous cleavage of NEXT by the aspartyl protease complex, γ-secretase, is the last step before an active Notch molecule is generated. As discussed briefly above, γ-secretase cleavage releases the intracellular domain (ICD) of many Type I proteins (Kopan and Ilagan, 2004), which can now travel to any cellular location for which they have the proper "zip code". Notch ICD contains two nuclear localization sequences flanking the ANK domain, thereby allowing it to enter the nucleus where it associates with the DNA-bound protein CSL converting it into an activator complex resulting in expression of target genes (reviewed in Barrick and Kopan, 2006; Lubman *et al.*, 2006).

The transmembrane domain of Notch1 is sufficient on its own to act as a γ-secretase substrate (Vooijs *et al.*, 2004), although the juxtamembrane regions from both sides of the membrane may factor in to modulate the efficiency of cleavage (Lee *et al.*, 2003; M.X.G.I and R.K., unpublished observations). NΔE and other direct substrates of γ-secretase are believed to bind to several docking sites on the γ-secretase complex (Kornilova *et al.*, 2006). While Nicastrin-mediated recognition does not distinguish between substrates, docking sites may add to the specificity of γ-secretase mediated cleavage of Notch and other substrates. Identification of other features in the transmembrane helices of substrates that might contribute to recog-

nition and specificity would be valuable to manipulate the γ-secretase complex during pathological states.

Once NΔE enters the active site, the transmembrane domain undergoes a series of γ-secretase mediated cleavage reactions. The cleavage site in Notch that results in the release of NICD had been mapped by microsequencing to the peptide bond between G1743 and V1744 (Fig. 2) (Schroeter *et al.*, 1998); a similar site (called ε-site) was mapped in APP in later studies (reviewed in Chapter 6). The S3/ε site is closer to the cytosolic face of the membrane whereas the γ-site is embedded deep within the lipid bilayer, initially fueling a controversy regarding the identity of the proteases involved in these seemingly distinct cleavage events. However, the observation that complete elimination of PS/γ-secretase activity inhibits generation of both ICD fragments and Aß-like peptides (Okochi *et al.*, 2002), and the demonstration that γ-secretase inhibitors designed to mimic the APP γ-cleavage site block APP proteolysis at the γ-sites and Notch proteolysis at S3/ε with an identical IC_{50} (De Strooper *et al.*, 1999; Lewis *et al.*, 2003; Schroeter *et al.*, 2003), suggested that a common activity mediates the proteolysis of both proteins. Interestingly, it is not as clear if inhibition at the S4/γ is inhibited with identical IC_{50} to inhibition at S3/ε (see below).

Immunoprecipitation of Notch C-terminal fragments followed by mass spectrometric analysis identified additional variants of NICD with diverse N-termini. Quantifying the amounts of these variants suggested that the predominant scissile bond lies between L1746 and S1747 (Okochi M., personal communication). Based on *in silico* predictions, S1747 is likely to be located outside the TMD. However, this and other NICD products with S, L or K at their amino terminus are sensitive to 26S proteasome-mediated degradation (Blat *et al.*, 2002; Varshavsky, 1996), reducing its abundance *in vivo* (Okochi M., personal communication). Interestingly, the choice of preferred scissile bond is determined by the localization of the γ-secretase/NEXT complex during cleavage. The preferred site of cleavage at the plasma membrane is indeed between G1743 and V1744, whereas cleavage in the early endosome generates peptides with the N-terminal residue S1747, perhaps due to differences in pH (Fukumori *et al.*, 2006; Okochi M., personal communication). It is important to note that reactivity to the neoepitope antibodies that recognize NICD with V1744 (as the amino terminal residue) frequently correlates with activation of Notch signaling, suggesting that V1744-NICD is perhaps the active molecule due to its stability, although we cannot rule out a role for other, short-lived products (Okochi M., personal communication). Interestingly, cleavage corresponding to S3 in other substrates, like APP, also accumulates ICD fragments with valine as the N-terminal residue. This can be readily explained by the relative susceptibility of different amino acids to N-end rule degradation (Varshavsky, 1996).

In addition to NICD, γ-secretase cleavage of NEXT also releases into the extracellular space Nβ peptides (Fig. 1B and 2), which are analogous to the Aß peptides produced from γ-site cleavage in APP (Okochi *et al.*, 2006). Mass spectrometric analysis reveals that as is the case with S3/ε, Aß /Nβ peptides are produced with varying length, each peptide terminating at different C-terminal residues (Lammich

et al., 2002), centered around a new Notch TMD cleavage site termed S4 located at the center of the transmembrane domain (Fig. 2) (Okochi *et al.*, 2002). Although most Nβ peptides end at A1731, other Nβ species with shorter and longer C-termini have been identified, much like the various Aβ species (Lammich *et al.*, 2002; Okochi *et al.*, 2002).

Diversity of cleavage site choices at S3 and S4 site suggests that γ-secretase does not recognize a sequence motif in its substrates. In addition, γ-secretase substrates do not share a common motif in their TMDs (Kopan and Ilagan, 2004). The lack of primary sequence similarity between γ-secretase substrates suggests that this enzyme may recognize a similar conformation, rather than recognizing the primary amino acid sequence. Accordingly, γ-secretase-mediated cleavage of the substrate TMD is minimally affected by changes in their amino acid composition. For example, phenylalanine scanning of the APP TMD shifted the scissile bond preferences of γ-secretase (Lichtenthaler *et al.*, 1999) rather than abolish cleavage. Mutagenesis of single residues around S4 to valine did not change the preferred site (between A1731 and A1732), although the frequency of cleavage at other positions did change (Tanii *et al.*, 2006). A phenylalanine scan of Notch has not yet been done.

The preferences of cleavage around S3 are a matter of some controversy. Replacing the sequence GCGV^{1744} to LLFF completely abolished activity (Brou *et al.*, 2000; Vooijs *et al.*, 2004). Point mutations at the P' position from V^{1744} to A, G, K and L did not completely abrogate cleavage (Chandu *et al.*, 2006; Vooijs *et al.*, 2004). Under steady state, NICD from NΔE harboring V1744G mutation is hardly detectable, resulting from a shift in the cleavage site to Leu1745 (Okochi M., personal communication). Upon treatment of cells with the proteasome inhibitor lactacystin, NICD with V1744G mutation accumulates (Blat *et al.*, 2002); attributing the drastic reduction of NICD accumulation from V1744G and V1744L mutants exclusively to N-end rule mediated degradation by the 26S proteasome. However, time course experiments using ^{35}S-Met demonstrated that conversion of N1-V1744G to NICD occurred at a slower rate relative to a wild type molecule (Chandu *et al.*, 2006). Similar results were also observed with V1774L. The accumulation of NEXT-like fragment when a V1744K mutation is inserted into an active form of a longer CD4-Notch hybrid protein lends additional support that S3 mutants are cleaved at a slower rate of cleavage (Mumm *et al.*, 2000). The ability of NΔE-V1744L to better compete with APP cleavage compared to NΔE can also be interpreted to suggest a longer association with the protease (Schroeter *et al.*, 2003). Interestingly, mutations at the S3 equivalent position in ErbB4 (V673I) also resulted in reduced ICD accumulation (Vidal *et al.*, 2005) and loss of function (Sardi *et al.*, 2006), however, isoleucine is also subject to N-end rule degradation. In contrast, S3/ε mutations in APP reduced AICD stability but did not cause decrease in the rate of AICD generation (Hecimovic *et al.*, 2004).

Independent from whether S3 mutations impact stability or proteolysis, the S3 site mutagenesis studies demonstrate that the function resides in the released ICD fragment and not in the intact protein (Huppert *et al.*, 2000; Sardi *et al.*, 2006).

Mouse embryos in which the V1744G substitution was knocked into the Notch1 locus demonstrated the requirement for a free NICD in Notch signaling (Huppert *et al.*, 2005; Huppert *et al.*, 2000). Interestingly, N1-V1774G supported somito-genesis, a Notch-dependent process, even in the absence of Presenilins. This suggests that in the absence of γ-secretase, N1-V1744G is cleaved by an unknown protease; perhaps due to the relaxation of the TMD helix often seen with Gly inser-tions. Indeed, using a tissue culture assay monitoring target activation following NICD translocation into the nucleus, we have demonstrated that N1-V1774G display slightly higher activity than N1 in presenilin-deficient fibroblasts (Huppert *et al.*, 2005). An independent study reported the presence of a yet to be cloned aspartyl protease, γ-three protease, that has the ability to cleave NΔE to release NICD (Crouthamel *et al.*, 2002).

3.3.   Is Intramembrane Proteolysis a Sequential Process?

S3 and S4 cleavage could be independent events; however, under steady-state conditions, none of the Nβ peptides that have been isolated extend to G1744, and conversely, none of the isolated NICD fragments have A1731 at its amino terminus. (The same is true for APP ε- and γ-cleavages. See below and Chapter 6.) This argues that the S3 and S4 cleavages either occur simultaneously or are interde-pendent processes. S4 may be cleaved first, followed by an amino- or endopeptidase trimming of the amino-terminus of the ICD. Inversely, cleavage may start at S3 and a carboxy- or endopeptidase activity would remove the intervening amino acids to generate Nß peptides. However, accumulating evidence suggest that γ-secretase mediates both intramembrane cleavages sequentially, with cleavage first occurring at S3 and followed by cleavage at S4.

First, we demonstrated that conventional aminopeptidases do not participate in trimming an S4 cleavage product to form NICD. Therefore, hydrolysis at S3 and S4 must both take place to produce Nß and NICD. Second, mutations at S3 that reduce release of NICD, also created a corresponding reduction in the hydrolysis at S4 site and in the amounts of Nβ released (Chandu *et al.*, 2006). This is consistent with a mechanism in which cleavage at S3 is a prerequisite to cleavage at S4. Third, had S4 cleavage been independent of S3, investigators should have been able to detect a longer NICD fragment under conditions where the proteasome is inhibited. Fourth, as we mentioned above, accumulation of NEXT when a V1744K mutation is inserted into an active CD4-Notch hybrid protein can only occur if S4 cleavage was also impaired (Mumm *et al.*, 2000).

In parallel to these studies, biochemical dissection of cleavages in the APP TMD has revealed a similar phenomenon. Analysis of the published data indicates that in APP, mutations around S3/ε reduce proteolysis at S4/γ (see Fig 5 in Lichtenthaler *et al.*, 1999; Tesco *et al.*, 2005). More importantly, several labora-tories identified proteolytic intermediates whose cleavage sites map between S3/ε and S4/γ of the APP TMD; one of these new sites was named ζ-cleavage (Zhao *et al.*, 2004). Longer Aβ peptides extending C-terminally until ζ-cleavage (Aβ1-46)

or beyond (Aβ1-48) were detected (Qi-Takahara *et al.*, 2005; Zhao *et al.*, 2005; Zhao *et al.*, 2004). Longer Aß peptides are consistent with cleavage occurring first at S3/ε and sequentially progressing towards S4/γ. Aβ1-46 and Aβ1-48 were converted into the shorter Aβ peptides (Aβ1-40 and Aβ 1-42, respectively) in the presence of γ-secretase; this process is sensitive to the γ-secretase inhibitor, DAPT. Precursor-product relationship was also established: with increasing concentration of inhibitor, levels of shorter fragments decreased while a concomitant increase was observed in the levels of corresponding peptides longer by three residues (Qi-Takahara *et al.*, 2005). Mass spectrometric analysis of Nβ peptides also identified longer forms, extending till Leu1736 (Okochi *et al.*, 2002). It is possible that longer Nβ peptides extending till G1743 might exist. In summary, γ-secretase appears to perform a series of sequential cleavages starting close to the cytosolic face of the substrate TMD and sequentially proceeding until the carboxy terminal charge overwhelms the hydrophobic forces holding the TMD in the membrane. The S4/γ sites reflect the point at which the peptides are expelled into the extracellular space.

It is intriguing to note that γ-secretase and other intramembrane proteases perform a hydrolytic reaction in a hydrophobic environment. The γ-secretase complex has a total of 19 transmembrane domains (9 for PS, 1 for Nicastrin, 2 for PEN-2 and 7 for Aph-1) with the active site aspartates deeply embedded in the membrane. Recently, two independent groups have deciphered low-resolution EM structures of the enriched complex. Although both groups obtained different sizes (300 kDa (Lazarov *et al.*, 2006) vs. ~1 Mda (Ogura *et al.*, 2006)) and shapes (round (Lazarov *et al.*, 2006) vs. heart-shaped (Ogura *et al.*, 2006)) for the intact complex, common insights were provided, which would aid in understanding the mechanism of the γ-secretase complex. Both structures suggest existence of a hydrophilic interior and two pores on either side of the transmembrane domain through which, probably, extracellular and intracellular products are released. The central hydrophilic pore may indicate the active site chamber of the γ-secretase complex where the actual cleavage reaction occurs. However, direct demonstration for existence of water around the active site was provided by cysteine scanning mutagenesis (Sato *et al.*, 2006; Spasic *et al.*, 2006). Based on the accessibility of cysteines to different sulfhydryl reagents, it was demonstrated that TMD 6 and TMD 7, which harbor the catalytic aspartates, form a hydrophilic pocket, and that the cytosolic-facing half of TMD 7 is most likely in an extended conformation and exposed to water throughout its length. Sato and colleagues (Sato *et al.*, 2006) suggested a funnel shaped pore with the catalytic aspartates forming a constriction; different inhibitors inhibited water accessibility to residues that may align the pore.

3.4. Role of Endocytosis in γ-secretase Cleavage: Location, Location, Location

In the early days of the hunt for γ-secretase, the debate addressing the location of this enzyme, relative to the cellular distribution of presenilin, was dubbed the

"spatial paradox". This apparent paradox was resolved by the finding that presenilin was also present at the plasma membrane, where Notch had to be in order for ligand to stimulate its cleavage (Kaether *et al.*, 2002; Ray *et al.*, 1999a). However, the precise location where γ-secretase cleaves Notch remains a subject of intense investigation. Recent observations suggest that post-shedding, mono-ubiquitination and endocytosis of Notch was required (Gupta-Rossi *et al.*, 2004). This was suggested to reflect the need for trafficking of the Notch/γ-secretase complex to an organelle where cleavage will occur although endocytosis was not required for cleavage of NEXT *in vivo* (Struhl and Adachi, 2000). Still, as described above, evidence is emerging that the site where cleavage occurs may have a bearing on the type of NICD molecules generated (Fukumori *et al.*, 2006). In addition, endosomal sorting has an important role in preventing improper Notch receptor activation. Mutations in ESCRT complex proteins vps25 or *erupted*/Tsg101/vps23 lead to accumulation of Notch in an endosomal vesicle, which surprisingly permits ectopic activation of Notch via γ-secretase-dependent proteolysis (Moberg *et al.*, 2005; Thompson *et al.*, 2005; Vaccari and Bilder, 2005). Since ligand also accumulates, it is unclear if this process represents *cis*-stimulation, shedding and intramembrane proteolysis or shedding-independent activity of γ-secretase. Another protein, Lethal (2) Giant Discs (LGD) is also required to maintain the OFF state of Notch; in its absence (or when it is over expressed), ligand-independent activation is seen (Childress *et al.*, 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006; Justice *et al.*, 2003). Interestingly, loss of *hrs* leads to Notch accumulation in an endosomal compartment upstream of the ESCRT or LGD complexes, preventing ectopic Notch activation (Childress *et al.*, 2006; Jaekel and Klein, 2006) and suggesting that the mis-trafficking of Notch may place it in a compartment where its proteolysis is less constrained either because Notch may exist in a permissive conformation or due to the existence of conditions that allow γ-secretase to cleave Notch without the benefit of ligand binding or shedding. It remains to be seen if γ-secretase is involved in LGD mediated activation. Therefore, ESCRT and LGD complexes are normally involved in Notch down-regulation, indicating that endosomal sorting could be a key to restricting activation to the cell surface and may contribute to pathogenesis in different cellular contexts. It is worth noting that the apical polarity protein *Crumbs* is also required to restrict the activity of γ-secretase and thus to limit the extent of Notch activation (Herranz *et al.*, 2006).

3.5.   γ-secretase Cleavage of Notch Ligands

Recently, several studies have shown that Notch ligands are also subject to extracellular cleavage by Kuzbanian/ADAM10, ADAM17/TACE metalloproteases (Bland *et al.*, 2003; Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six *et al.*, 2004) and potentially other ADAM proteases (Dyczynska *et al.*, 2006; Sapir *et al.*, 2004) followed by γ-secretase cleavage of their TMDs. While ligand proteolysis was shown to be constitutive (Six *et al.*, 2003), it can also be induced by Notch

binding (Bland *et al.*, 2003). Ligand processing is thought to be important for its downregulation and membrane clearance, which helps promote and maintain unidirectional signaling (Sapir *et al.*, 2004) or alleviate *cis*-inhibition (Mishra-Gorur *et al.*, 2002). Alternatively, ligand proteolysis could be releasing biologically active fragments, e.g. soluble ligands that could potentially positively or negatively regulate Notch signaling events (Hukriede and Fleming, 1997) and/or ligand ICD fragments (analogous to NICD). ICD fragments could be involved in a bi-directional mode of signaling for Notch; however, a signaling function for ligand ICDs remains to be demonstrated in a biologically relevant system. Additional studies will also have to address whether ligand cleavage is required for Notch signaling function. It is clear that for most Notch mediated decisions, γ-secretase deficient cells can signal well (Chung and Struhl, 2001; Lopez-Schier and Johnston, 2002).

4. CONCLUSIONS AND PERSPECTIVES

In elucidating the mechanisms of Notch signaling, modern investigators benefited greatly from the myriad of genetic studies on the *Drosophila* Notch locus as well as its *C. elegans* homologs GLP-1 and LIN12 to complement cell culture-based approaches. Notch-related disease processes also provided significant mechanistic clues. Because intramembrane proteolysis is central to the Notch signaling mechanism and the biological outcomes of this pathway are sensitive to dose, timing and cellular context, studying γ-secretase from the Notch perspective will continue to contribute to our understanding of how γ-secretase activity is temporally and spatially regulated.

The emerging picture of γ-secretase is that of a promiscuous enzyme that seems to cleave many, if not all, type I membrane proteins after they have undergone ectodomain shedding. It will be interesting to see how many other cellular processes are controlled by γ-secretase mediated intramembrane proteolysis; the same question applies to all I-CLiPs. An unknown fraction of substrates engage in signaling after being 'RIPped'. For instance, in contrast to Notch and Erb4, it appears that RIP of APP terminates a function (Hass and Yankner, 2005). It is likely that most substrates undergo intramembrane proteolysis simply to remove the TMD from the lipid membranes. The challenge will therefore lie in delineating functions of γ-secretase cleavage itself (to terminate or activate a process) from the functions of its cleavage product(s).

At the mechanistic level, we hope that soon advances in crystallography and NMR will permit the study of γ-secretase in a complex with Notch with atomic resolution. Such biophysical studies will be necessary to resolve many of the mechanistic models proposed for the action of I-CLiPs in general and γ-secretase in particular. The first such studies were recently reported and these immediately stimulated a new debate rather then settle an old one (Urban, 2006; Wang *et al.*, 2006; Wu *et al.*, 2006), indicating that the field will remain active and vibrant for sometime to come.

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